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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b>  The focus of this study is to understand the biology behind the metastasis suppression via BRMS1, a recently identified metastasis suppressor gene. BRMS1 is a protein with a glutamic acid rich N-terminus, coiled-coil domain, an imperfect leucine zipper and nuclear localization signals. It is expressed almost ubiquitously in human tissues and is highly conserved across species. Sub-cellular fractionation and fluorescence immuno-cytochemistry has indicated that it localizes to nucleus. BRMS1 is shown to restore homotypic gap-junctional communication. Our hypothesis is that it may be involved in transcription regulatory complex.  To identify proteins that interacting with BRMS1 a yeast two-hybrid screen was performed using full length BRMS1 as a bait and human mammary gland library as a prey. We confirmed RBP1 (Rb binding protein), FLJ00052 (EST), MRJ (Hsp40 related chaperon) and Nmi (N-myc interactor) as potential interactors at cellular level by co-immunoprecipitation studies. We have further demonstrated that BRMS1 is a component of mSin3-HDAC complex. Based on these observations it is tempting to speculate that BRMS1 regulates gene expression by histone deacetylation. Currently we are studying the role of this complex in regulation of metastasis of breast cancer				
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b) Cancer letters (in preparation)	
c) Cancer letters (in preparation)	

## Summary Report July 2004

### Introduction

The proposed work comprises a single specific aim of mutagenizing Breast Cancer Metastasis Suppressor 1, BRMS1, for establishing its mechanism of action. The basic work is broadly divided into two parts (a) Identification of interacting protein(s) (b) Mutational analysis.

Identification of protein(s) interacting with BRMS1 involves

- Screening of "prey" library to identify the possible interactors.
- Test the effect of critical mutations identified by mutational analysis on the protein-protein interaction.

The mutational analysis involves

- Constructing BRMS1 deleted for predicted domains and testing the effect of deletions *in vitro* and *in vivo*.
- Construction of site directed mutations and testing them *in vivo* and *in vitro*.

Both these parts were proposed to be carried out simultaneously and in this report the progress on both fronts is summarized.

Until June 2003 the project had following Key Accomplishments :

- ✓ Yeast two hybrid screen for protein interactors of BRMS1 is successfully performed
- ✓ Eight genetic interactors were discovered.
- ✓ RBP1 and MRJ interactions were verified with co-immunoprecipitation
- ✓ Assays of *in vitro* characterization of BRMS1 were standardized.
- ✓ BRMS1 interaction with NMI confirmed
- ✓ BRMS1 mSDS3 interaction confirmed
- ✓ Discovered BRMS1 as one of the member of a ~1.6 MDa mSin3-HDAC complex.
- ✓ Generated specific internal deletions in BRMS1 to disrupt various domains.

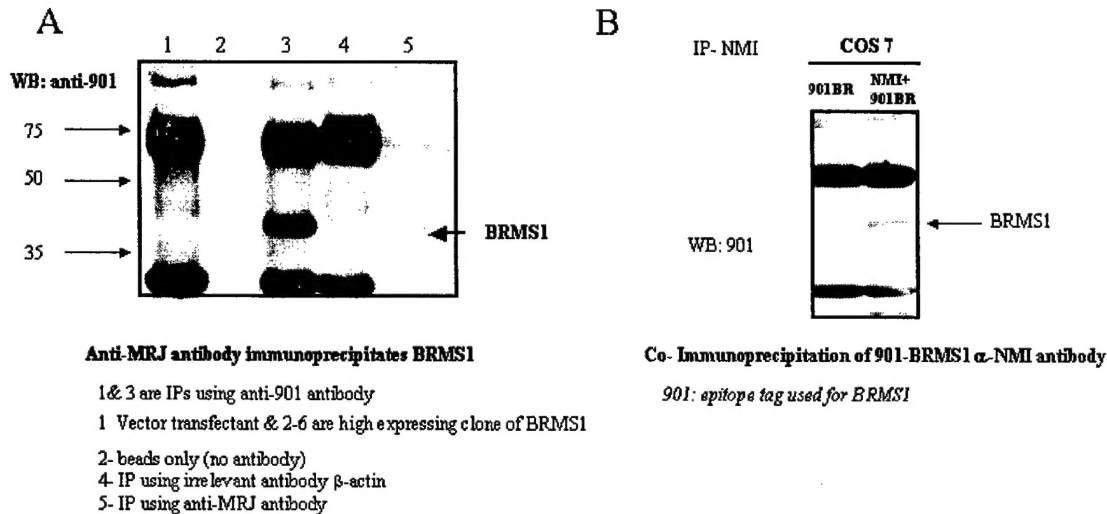
### Summary of the work:

#### ***Identification of interactors of BRMS1***

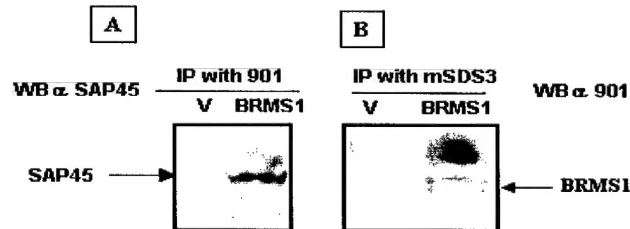
Prey libraries from three different human tissues *viz.* breast, placenta and prostate were screened. The breast library was chosen based on the fact that BRMS1 was identified based on studies on metastatic breast carcinoma cell lines and was functionally shown to block the metastasis of breast cancer cell lines in nude mouse model. Placenta and prostate are tissues that express the highest levels of BRMS1. The screen was performed using full length BRMS1 as a bait. The results of these screens are summarized in Table1 (numbers indicate independent clones). Eight genetic interactors of BRMS1 were identified. These are RBP1 (Rb binding protein), MRJ (Hsp40 related chaperone), CCG1 (a protein essential for progression of G phase), SMTN (cytoskeletal protein specific to smooth muscles), FLJ00052 (EST), Nmi (N-myc interactor), KPNA5 (karyopherin alpha 5), and BAF 57(BRG1 associated factor). The BRMS1-RBP1 (please refer to attached J. Biol. Chem. manuscript), BRMS1-MRJ as well as BRMS1-



NMI interactions were further confirmed at cellular level by co-immunoprecipitation studies (Figure 1 a, b). Further characterization of the immunoprecipitated complex using HPLC, co-immunoprecipitation and Western blotting, suggests BRMS1 is a member of mSin3-HDAC complex. [Manuscript accepted in J. Biol. Chem.].



Interestingly the EST, FLJ00052, discovered by us in the yeast two hybrid screen as coding for a possible interactor for BRMS1 is published recently as mSDS3 or SAP45 (Fleischer *et al*, Alland *et al*). mSDS3 is also shown to be a member of mSin3-HDAC complex . We have



**Fig 2.** A: Immunoprecipitation of 901 tagged BRMS1 also pulls down Msds3  
 B: Immunoprecipitation of mSDS3 pulls down Msds3  
 These studies were carried out in 901 tagged BRMS1expressors of MDA-MB-231

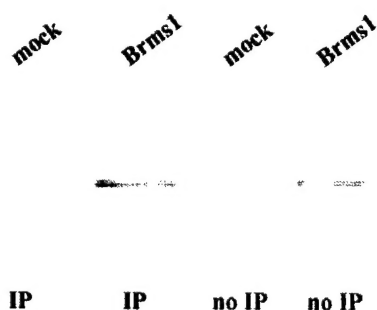
confirmed the BRMS1-mSDS3 interactions using the antibodies kindly provided by Don Ayer Huntsman Cancer Institute Department of Oncological Sciences, Salt Lake City, UT. (Fig 2 a & b). mSDS3 shares 24 %identity and 50% similarity with BRMS1. Based on GenBank searches performed by us and others MGC11296 is another EST that is possibly coding for a stronger ~60 % homolog of BRMS1. Thus we have found a family of BRMS1-proteins.

Our finding that BRMS1 interacts with mSDS3 and is a part of the mSin3-HDAC complex combined with other groups finding that mSDS3 forms a part of the same complex imply strongly that BRMS1 and mSDS3 are present together in the same complex. It is intriguing to see the BRMS1 family (homologs) are present in a complex that regulates gene expression. Based on the literature search, there are no major findings reported yet regarding which

downstream genes are regulated by this complex. But it is tempting to speculate that this is a complex that plays a major role in control of the metastatic phenotype. Experiments are underway to address this question.

We have performed forward as well as reverse co-immuno precipitations to confirm the interaction of BRMS1 with RBBP1, NMI, MRJ, SDS3 and BAF57 (Copy of Manuscript in preparation attached). The HDAC complexes and the other chromatin remodeling complexes such as SWI-SNF complexes are speculated to be working together. In a series of co-immunoprecipitation studies, we have observed that BRMS1 interacts with class I as well as class II HDAC members. (Manuscript in preparation, copy attached). We have also found that some of the interactors are able to co-immunoprecipitate each other. Specifically SDS3 co-immunoprecipitates BAF 57 (data in attached manuscript). This suggests that the HDAC and SWI-SNF complexes are working in close physical proximity, regulating a set of genes presumably important in metastasis.

Murine ortholog of BRMS1 is 95% identical to human BRMS1. Hence to strengthen our finding of BRMS1-HDAC interaction, we evaluated murine *brms1* for interaction with murine HDAC1 and found that the interaction was reproducible in murine systems as well (Figure 3).



**Figure 3. Murine HDAC1 co-immunoprecipitates Brms1.**

Myc-Brms1 transfected 4T1 cells were compared to mock transfected (mock, no DNA). The first two lanes were immunoprecipitated with anti-HDAC1 Ab and the last two lanes contain only cell lysate. The blot was probed with anti-Myc Ab. The band corresponds to a MW of Brms1 i.e. near 35 kDa.

*We had proposed that we will perform BRMS1 mutagenesis to see loss of the metastasis suppression function. We have decided to use this strategy in a slightly modified way. We will first find the mutant(s) that abrogates the participation of BRMS1 in the mSin3-HDAC complex. This mutant(s) will then be evaluated for loss of metastasis suppression ability. The mutants generated are described below.*

#### **Mutational analysis:**

We had previously inspected BRMS1 for conserved domains and found various domains such as coiled-coil domain, nuclear localization sequences, N-terminal glutamic acid rich region and an imperfect leucine zipper. We looked at the BRMS1 protein sequence again in the light of the Y2H results, using the Pfam conserved domain search provided by NCBI. Many interesting conserved domains were observed. Many of them overlap and are disrupted in the BRMS1 deletion performed by us as explained below. We have used the ExSite<sup>TM</sup> inverse PCR strategy to create specific internal deletions of BRMS1.

- ▶ Deletion of Glutamic acid rich region BRMS1ΔE: deletion of aa.11 to 63
- ▶ Deletion of Coiled coil domain BRMS1 ΔC: deletion of aa. 67 to 87
- ▶ Deletion of Leucine zipper region BRMS1 ΔL: deletion of aa. 138 to 181

We have also independently mutated the two NLS sequences to alanine and also have both the NLS mutated to alanine. All these constructs have N-terminal c-Myc tag for the ease of performing co-immunoprecipitations.

We are also using yeast reverse two hybrid screen using RBP1 as a bait to obtain mutated BRMS1 that would fail to interact. Both the approaches are expected to yield specific amino acids required for BRMS1 to be a member of the mSIN3-HDAC complex. These mutants will then be evaluated in animals for metastasis suppression. If the formation of the complex is relevant to metastasis suppression, mutated BRMS1 will lose that capability.

#### **Key Accomplishments in 2003-2004:**

- BRMS1 interaction with BAF57 confirmed
- BRMS1 was found to be binding to Class I as well as Class II HDACs.
- Murine BRMS1 was found to interact with murine HDAC1 further confirming the BRMS1-HDAC interaction.
- This work was chosen for **presentation in 2004 AACR-Mini Symposium Lecture** "Characterizing protein-protein interactions of breast cancer metastasis suppressor 1 (BRMS1)". Proceedings of the American Association for Cancer Research (2004) 45: 2392 Orlando, FL.

#### **Note:**

- ★ It is essential to mention that due to the move of the Mentor, Professor Danny R. Welch to Univ. of Alabama at Birmingham, the grant had to be moved from Hershey Medical Center, PA, to UAB, Birmingham. The move of funds was very slow and actually the funds are received in UAB very recently. It must emphasized that despite of this fact concerted efforts have been made to accomplish the specific aims and pursue important leads emerging from the research finding.
- ★ The P.I. has accepted a position as Assistant Professor in the University of South Alabama, effective June 2004. P. I. has been chosen as a key faculty in the Metastasis and Tumor Biology Center of the new Cancer Center at the University of South Alabama.

#### **Reportable Outcomes:**

##### **Publications in Peer reviewed journals**

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### Abstracts

- ▶ Shevde LA, **Samant RS**, Casey G and Welch DR. BRMS1 suppresses expression of osteopontin. *9<sup>th</sup> international congress of the Metastasis Research Society*, Chicago, September 20-22, 2002.
- ▶ **Samant RS**, Meehan WJ, Shevde LA, Hopper JE, Welch DR. Identifying protein interactors of Breast Cancer Metastasis Suppressor 1, BRMS1. *9<sup>th</sup> international congress of the Metastasis Research Society*, Chicago, September 20-22, 2002.
- ▶ **Samant RS**, Meehan WJ, Shevde LA, Hopper JE, Welch DR. Identifying protein interactors of Breast Cancer Metastasis Suppressor 1, BRMS1. *Era of Hope*, 2002. CDMRP meeting of the U.S. Army Materiel Command, Florida, September 24-28, 2002.
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- ▶ Meehan, W.J., **Samant, R.S.**, Hopper, J.E., Carrozza, M.J., Shevde, L.A., Workman, J.L., Eckert, K.A., Verderame, M.F., and Welch, D.R. BRMS1 (breast cancer metastasis suppressor1) forms complexes with RBP1 and mSin3 histone deacetylase complex and represses transcription. 5<sup>th</sup> international congress of the Genetics, Biochemistry and Physiology of NDP kinase/NM23 /AWD. T19.
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- ▶ **Samant, R.S.**, Meehan, W.J., Mehta, A., Shevde, L.A., and Welch, D.R., Characterizing protein-protein interactions of breast cancer metastasis suppressor 1 (BRMS1). *Proceedings of the American Association for Cancer Research* (2004) 45: 2392.

### Conclusion:

Eight protein interactors of BRMS1 have been identified. At least 4 of them are confirmed by forward as well as reverse co-immunoprecipitation. The project lead to the discovery that BRMS1 is a member of mSin3-HDAC complex. We also found that BRMS1 has closely related family members and at least one of them interacts with BRMS1 and possibly is a part of the same HDAC complex. Murine Brms1 also interacts with murine HDAC1 hence the function of BRMS1 is conserved across species. BRMS1 was found to interact with Class I and Class II HDACs. Since one of the interactors (BAF 57) is a member of SWI-SNF (Chromatin remodeling complex), BRMS1 is possibly involved in working closely with HDACS and SWI-SNFs and possibly a molecular bridge. **Considering that HDAC inhibitors are recently in clinical trials, these finding have direct clinical relevance. Hence investigations of the BRMS1 and it's interacting proteins for involvement in regulatory protein complex(es) and the downstream regulatory pathways should be a very high priority research and has great potentials in unrevealing critical events in metastasis.**

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## Breast Cancer Metastasis Suppressor 1 (BRMS1) Forms Complexes with Retinoblastoma-binding Protein 1 (RBP1) and the mSin3 Histone Deacetylase Complex and Represses Transcription\*

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**Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis of multiple human and murine cancer cells without inhibiting tumorigenicity. By yeast two-hybrid and co-immunoprecipitation, BRMS1 interacts with retinoblastoma binding protein 1 and at least seven members of the mSin3 histone deacetylase (HDAC) complex in human breast and melanoma cell lines. BRMS1 co-immunoprecipitates enzymatically active HDAC proteins and represses transcription when recruited to a Gal4 promoter *in vivo*. BRMS1 exists in large mSin3 complex(es) of ~1.4–1.9 MDa, but also forms smaller complexes with HDAC1. Deletion analyses show that the carboxyl-terminal 42 amino acids of BRMS1 are not critical for interaction with much of the mSin3 complex and that BRMS1 appears to have more than one binding point to the complex. These results further show that BRMS1 may participate in transcriptional regulation *via* interaction with the mSin3-HDAC complex and suggest a novel mechanism by which BRMS1 might suppress cancer metastasis.**

The complex process of cancer cell dissemination and the establishment of secondary foci involves the acquisition of multiple abilities by metastatic cells. For example, blood-borne metastasis requires cells to invade from the primary tumor, enter the circulation, survive transport, arrest at a secondary site, recruit a blood supply, and proliferate at that site (1). The ability to accomplish all of these steps likely involves changes

in, and coordinated expression of, a large assortment of genes. Consistent with this notion, several genes, proteins, and pathways have been associated with metastatic progression, including oncogenes, motility factors, and matrix metalloproteinases (1, 2). In addition to metastasis-promoting genes, a new class of molecules called metastasis suppressors has been described (reviewed in Refs. 2–5). By definition, metastasis suppressors inhibit metastasis without blocking primary tumor growth, presumably by inhibiting one or more steps necessary for metastasis. To date, 13 metastasis suppressor genes have been identified that reduce the metastatic ability of cancer cell line(s) *in vivo* without affecting tumorigenicity, namely breast cancer metastasis suppressor 1 (BRMS1),<sup>1</sup> CRSP3, DRG1, KAI1, KISS1, MKK4, NM23, RhoGDI2, RKIP, SSeCKs, VDUP1, E-cadherin, and TIMPs (reviewed in Refs. 4 and 5).

We identified *BRMS1* using differential display to compare highly metastatic breast carcinoma cells with related but metastasis-suppressed cells (6). Enforced expression of *BRMS1* suppressed metastasis in three animal models, namely human breast (6), murine mammary (7), and human melanoma cells (8). Additionally, *BRMS1* mapped to loci in murine (7) and human (6) genomes that had previously been implicated in metastasis control (9). The *BRMS1* protein localized to nuclei and restored gap junctional intercellular communication in both breast and melanoma tumor cell lines (8, 10, 11), but its molecular functions remain to be elucidated.

One approach to determine a mechanism of action involves identifying which proteins interact with *BRMS1*. In this report, we utilized yeast two-hybrid and co-immunoprecipitation (co-IP) to demonstrate that *BRMS1* interacts with retinoblastoma-binding protein 1 (RBP1). This association led to experiments to demonstrate that *BRMS1* interacts with at least seven members of the mammalian Sin3 (mSin3) mSin3-histone deacetylase (HDAC) complexes, including HDAC1 and HDAC2.

Human HDACs exist in many large, multi-subunit protein complexes (12) that are recruited to specific regions by DNA-binding factors. As their name indicates, HDACs remove acetyl groups from lysine residues at the N-terminal tails of core

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) XM 045014.

<sup>b</sup> These authors contributed equally to this work.

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<sup>1</sup> The abbreviations used are: BRMS1, breast cancer metastasis suppressor gene 1; co-IP, coimmunoprecipitation; RBP1, retinoblastoma binding protein 1; HDAC, histone deacetylase; mSin3, mammalian Sin3 (suppressor of defective silencing 3); NuRD, nucleosomal remodeling and deacetylation; Rb, retinoblastoma; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoic acids and thyroid hormone receptor.

histones (13–15). Histone deacetylation favors transcriptional repression, whereas acetylation (mediated by histone acetyltransferases) favors transcriptional activation. mSin3-HDAC complexes are named for the large mSin3A and mSin3B proteins, which are thought to serve as scaffolds for complex assembly (14). HDAC enzymatic activity in mSin3 complexes is mediated by a core subunit consisting of HDAC1, HDAC2, RbAp46, and RbAp48 (13). The core HDAC subunit is also found in at least one other HDAC complex, NuRD (nucleosomal remodeling and deacetylation) (16).

Mammalian Sds3 (mSds3; suppressor of defective silencing 3) was recently reported to be an integral component of the mSin3 complex and acts to stabilize HDAC1 within the complex (17). BRMS1 shares homology with mSds3, suggesting that BRMS1 belongs to a protein family (17). mSin3-associated proteins, SAP18 and SAP30, which are believed to serve as adapter molecules, complete the core complex as currently understood (18–20).

#### MATERIALS AND METHODS

**Cell Lines, Cell Culture, and Transfections**—MDA-MB-231 is a human estrogen receptor- and progesterone receptor-negative cell line derived from a pleural effusion from an infiltrating ductal breast carcinoma. C8161 is a metastatic, amelanotic human melanoma cell line derived from an abdominal wall metastasis. C8161.9 is a highly metastatic clone obtained by limiting dilution cloning of C8161 (21). 66cl4 is a murine mammary carcinoma cell line derived from a spontaneous carcinoma in BALB/c3H mice (22, 23). All cell lines were cultured in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia), 1% non-essential amino acids, and 1 mM sodium pyruvate. Transfected cells also received 500  $\mu$ g/ml G418 (Geneticin; Invitrogen). All cells were maintained on 100-mm Corning tissue culture dishes at 37 °C with 5% CO<sub>2</sub> in a humidified atmosphere. MDA-MB-231 cells were passaged at 80–90% confluence using a solution of 0.125% trypsin and 2 mM EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Dulbecco's phosphate buffered saline (CMF-DPBS). C8161.9 and 66cl4 cells were passaged at 80–90% confluence using 2 mM EDTA in CMF-DPBS. BRMS1 was cloned into the constitutive mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) under control of the cytomegalovirus promoter. No antibiotics or antimycotics were used. All cell lines were found to be negative for *Mycoplasma* spp. contamination using a PCR-based method (TaKaRa, Madison, WI).

To detect BRMS1 protein expression, a chimeric molecule was constructed with an N-terminal epitope tag (SV40T epitope 901) (24, 25). Epitope-tagged full-length BRMS1 and deletion mutants were cloned into pcDNA3 before introduction into cells by electroporation (Bio-Rad model Gene Pulser; 220 V, 960 microfarads,  $\infty$  ohms). Briefly, cells (0.8 ml;  $1 \times 10^7$  cells/ml) from 80% confluent plates were detached, plasmid DNA (10–40  $\mu$ g) was added to the cells, and the mixture was placed onto ice for 5 min before electroporation, followed by 10 min on ice prior to plating on 100-mm cell culture dishes. Transfectants were selected using G418 (Geneticin; 500  $\mu$ g/ml). Single-cell clones were isolated by limiting dilution in 96-well plates. Stable transfectants were assessed for protein expression by immunoblotting.

**Constructs**—Deletion mutants were created by unidirectional digestion with exonuclease III as described previously (26). Briefly, pcDNA3 901-BRMS1 was digested by Apal and Bsu36I in the 3' multiple cloning site and then digested with 150 units/pmol DNA exonuclease III (Promega) at 37 °C. Reactions were stopped at different time points to create a nested set of C-terminal BRMS1 deletion mutants. Sequencing confirmed that the following 3' deletion mutants were successfully created: 1) 901-BRMS1( $\Delta$ 204–246) + LFYSVT; 2) 901-BRMS1( $\Delta$ 164–246) + TIL; and 3) 901-BRMS1( $\Delta$ 91–246) + FYSVT. Additional amino acids were added because a short stretch of vector DNA was transcribed prior to encountering a stop codon. Hereafter, these constructs will be designated BRMS1( $\Delta$ 204–246), BRMS1( $\Delta$ 164–246), and BRMS1( $\Delta$ 91–246), respectively.

**Antibodies**—An antibody directed against the 901 epitope was generously provided by Dr. Satvir Tevethia. Anti-MTA1 was a gift from Dr. Garth Nicolson. Anti-RBP1 (clone LY32 and initial aliquots of clone LY11) were gifts of Dr. Philip Branton. Antibodies directed against HDAC1, HDAC3, NCoR, RBP1 (clone LY11), SAP30, mSin3A, and SMRT were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies recognizing E2F and retinoblastoma (Rb) were bought from

Pharmingen. Antibodies directed against HDAC2, Mad1, Max, Mi-2, p107, p130, RbAp46, RbAp48, SAP18, and mSin3B were obtained from Santa Cruz Biotechnology.

**Yeast Two-hybrid Screen**—A yeast two-hybrid screen was performed to isolate cDNAs encoding BRMS1-interacting proteins essentially as described in the manufacturer's instructions (Clontech MATCHMAKER LexA). Full-length BRMS1 was cloned in-frame with the GAL4 DNA binding domain in the pDBTrp (Invitrogen) vector to obtain pDB-BRMS1. This GAL4DB-BRMS1 fusion (bait) construct was used to transform AH 109 (*MATa, trp-901, leu2-3, 112, ura3-52, his3-200, gal4 $\Delta$ , gal80 $\Delta$ , LYS2::GAL<sup>UAS</sup>-GAL1<sup>TATA</sup>-HIS3, GAL2<sup>UAS</sup>-GAL2<sup>TATA</sup>-ADE2, URA3::MEL1<sup>UAS</sup>-MEL1<sup>TATA</sup>-lacZ, MEL1*). Human breast, prostate, and placenta cDNA libraries in pACT2 (MATCHMAKER, BD Biosciences Clontech) were screened in yeast drop-out minimal medium lacking histidine, tryptophan, and leucine. His<sup>+</sup> colonies were tested for growth on minimal medium lacking adenine, tryptophan, leucine, and  $\beta$ -galactosidase activity as described previously (27). cDNA plasmids were isolated from each positive yeast clone using Zymoprep (Zymo Research, Orange, CA) and sequenced. The interaction phenotype was lost when either the bait or prey plasmid was lost from the cell. Re-introduction of missing partners restored growth on minimal medium lacking histidine, tryptophan, and leucine, growth on medium lacking adenine, tryptophan and leucine, and restoration of  $\beta$ -galactosidase activity.

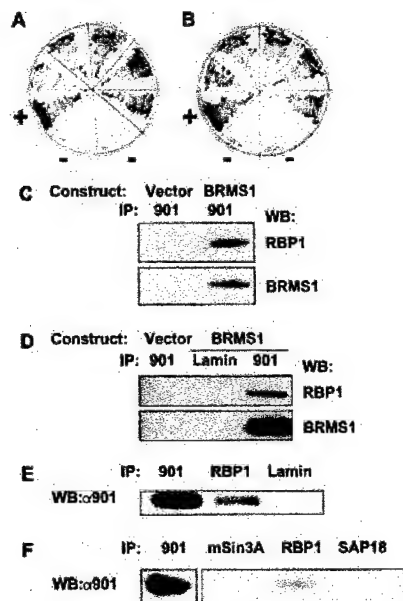
**<sup>35</sup>S Protein Labeling**—Cells were grown to 80–90% confluence in 100-mm tissue culture plates. Media were removed and replaced with 3 ml of cysteine-methionine-free media (Invitrogen) containing 5% fetal bovine serum for 1 h. Media were removed and replaced with 3 ml of cysteine- and methionine-free media containing 5% fetal bovine serum and 100  $\mu$ Ci/ml <sup>35</sup>S-express protein labeling mix (PerkinElmer Life Sciences). Cells were incubated for 18 h before protein was collected for co-IP.

**Co-immunoprecipitation**—Cells (90–95% confluence) were washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (0.5% Igepal CA-630 (Sigma), 50 mM Tris, pH 8, 150 mM NaCl, and 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 50 mM NaF, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, and 10  $\mu$ l/ml of a protease inhibitor mixture containing 4-(2-aminoethyl) benzensulfonylfluoride (AEBSF), pepstatin A, *trans*-epoxysuccinyl-L-leucylamido(4-guanido)butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Lysate was kept at 4 °C during all subsequent steps. Lysate was passed through a 21-gauge needle several times, incubated on ice for 1 h, then centrifuged for 1 h at 12,000  $\times g$  in a Sorvall MC 12V microcentrifuge with an F12/M.18 rotor to remove insoluble debris. Lysates were then rocked gently in the presence of antibody for 1 h, followed by the addition of 20  $\mu$ l of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) and rocking overnight. Agarose beads were washed twice with ice-cold PBS, heated to 60 °C in sample buffer, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membrane for immunoblotting. For <sup>35</sup>S-labeled samples, films were exposed directly to polyvinylidene difluoride membranes. In each experiment, blots were probed with antisera to the immunoprecipitated protein to verify the quality of the immunoprecipitation and assess the equal loading of lanes.

**Size Exclusion Chromatography**—Whole cell protein lysate (pooled from 10 100-mm plates using 1 ml of lysis buffer each) was applied to a Superose 6 HR 10/30 size exclusion column (Amersham Biosciences). The column was run using lysis buffer with 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol at a flow rate of 0.2 ml/min. Fractions (500  $\mu$ l) were collected, and 420  $\mu$ l of each fraction were used for co-IP. The remaining 80  $\mu$ l was used for immunoblotting.

**HDAC Activity Assay**—Following co-IP, agarose beads were combined with 400  $\mu$ l of HDAC assay buffer (15 mM Tris, pH 7.9, 10 mM NH<sub>4</sub>Cl, 0.25 mM EDTA, 10% glycerol, and 10 mM  $\beta$ -mercaptoethanol) containing 1.5  $\mu$ g <sup>3</sup>H-labeled chicken reticulocyte core histones (28) with or without 250 mM sodium butyrate (an HDAC inhibitor). Samples were inverted continuously on a rotating wheel for 3 h at 30 °C, and HDAC activity was measured as described previously (28). Briefly, the reaction was stopped by adding 100  $\mu$ l of 1 M HCl/0.4 M acetic acid and 0.8 ml ethyl acetate. Samples were vortexed for 30 s and centrifuged at 8,000  $\times g$  for 5 min. An aliquot (0.6 ml) of the upper (organic) phase was then counted for radioactivity in a 5-ml scintillation mixture (Fisher).

**Reporter Assays**—BRMS1 cDNA was cloned in-frame with the N-terminal Gal4-DNA binding domain in pBIND (Promega). Subconfluent (80–90%) COS7 cells were transfected using the FuGENE reagent (Roche Diagnostics) with the GAL4-BRMS1 fusion construct and a luciferase reporter plasmid containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, kindly provided by Dr. Ron Eisenman. pRLSV40 (*Renilla* luciferase) was used as a transfection control. Trichostatin A (50, 150, and 300 ng/ml, Sigma)



**Fig. 1. Yeast two-hybrid and co-IP indicate that BRMS1 interacts with RBP1.** *A*, growth of representative positive candidates on minimal media lacking histidine, tryptophan, and leucine. *B*, growth of representative positive candidates on minimal medium lacking adenine, tryptophan, and leucine. Plus sign (+) indicates positive control, minus sign (−) indicates negative control (AH109 with BRMS1 or interactor cDNA alone). *C*, BRMS1 co-immunoprecipitated RBP1 from whole cell lysate (1 mg) in MDA-MB-231 cells. Anti-901 was used to immunoprecipitate (IP) epitope-tagged BRMS1 and also pulled down RBP1, as shown by Western blot (WB). Anti-901 did not pull down RBP1 in vector-transfected cells. *D*, BRMS1 co-immunoprecipitated RBP1 from whole cell lysate (1 mg) in C8161.9 cells. Anti-901 was used to immunoprecipitate epitope-tagged BRMS1 and also pulled down RBP1, as shown by Western blot. Anti-901 did not pull down RBP1 in vector-transfected cells, and anti-Lamin A/C (an irrelevant antibody) did not pull down RBP1 in BRMS1-transfected cells. *E*, anti-RBP1 co-immunoprecipitated BRMS1 in MDA-MB-231 cells. Immunoblotting with  $\alpha$ -901 was used as a positive control, and  $\alpha$ -Lamin A/C was used as a negative control. *F*, anti-RBP1 co-immunoprecipitated BRMS1 in C8161.9 cells. Immunoblotting with  $\alpha$ -901 was used as a positive control, and  $\alpha$ -mSin3A and  $\alpha$ -SAP18 were used as negative controls.

was added for 24 h prior to lysis. Cells were lysed in Passive lysis buffer (Promega) 48 h post-transfection. Cell extracts were assayed for luciferase activity using the Dual luciferase reporter assay system (Promega) and an automated luminometer Monolight™3010 (Pharmingen). Transfection efficiencies were normalized using the *Renilla* luciferase control.

## RESULTS

**RBP1 and mSds3 Were Identified as BRMS1-interacting Proteins by Yeast Two-hybrid Screen**—A yeast two-hybrid screen was performed using prey libraries from three human tissues, breast, placenta, and prostate. Breast was chosen because BRMS1 was first identified as a metastasis suppressor in breast cancer. Placenta and prostate were chosen because BRMS1 mRNA is highly expressed in these tissues (6). Full-length BRMS1 was used as the “bait.” RBP1 was present in the majority of positive clones from breast and placenta libraries, so it was chosen for further studies (Fig. 1, *A* and *B*).

The FLJ00052 expressed tag was present as two independent positive clones in a prostate library screen. During the completion of the work reported here, FLJ00052 was identified as the mammalian ortholog (mSds3, GenBank™ accession number XM\_045014 mapping to human chromosome 12q24.23) of the yeast Sds3 protein. There are other related genes according to the LocusLink ([www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=FLJ00052&ORG=&V=0](http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=FLJ00052&ORG=&V=0)), suggesting the existence of additional mSds3 orthologs. mSds3 is an integral

component of the mSin3-HDAC co-repressor complex, modulates HDAC activity, and stabilizes the complex (17). Antibodies recognizing mSds3 are not available commercially; thus, we have not yet been able to test whether BRMS1 pulled down mSds3.

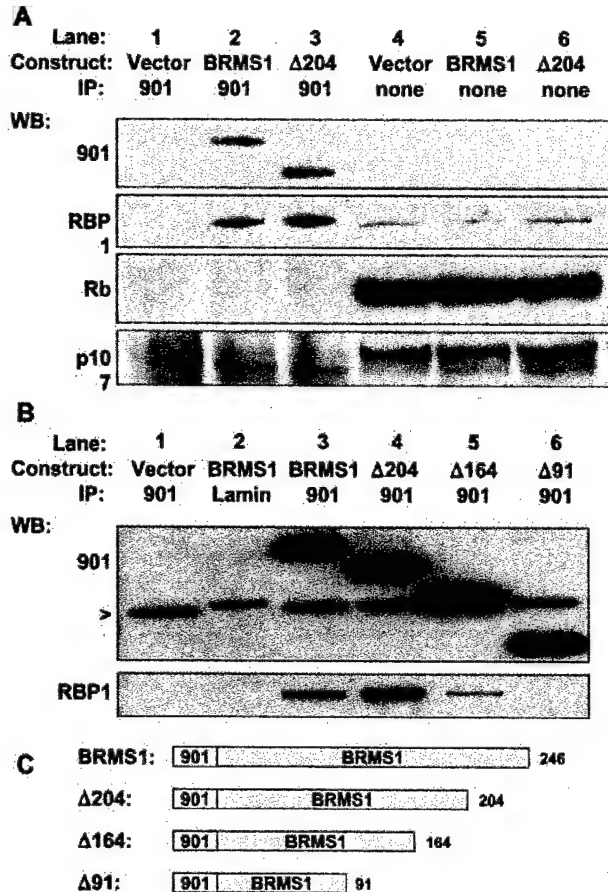
**BRMS1 and RBP1 Are Reciprocally Co-immunoprecipitated in Human Breast and Melanoma Cancer Cells**—MDA-MB-231 human breast carcinoma cells and C8161.9 human melanoma cells were transfected with 901 epitope-tagged BRMS1. Immunoprecipitation of BRMS1 followed by immunoblot with two RBP1-specific antibodies (clones LY11 and LY32) (Fig. 1, *C* and *D*) showed that BRMS1 co-immunoprecipitates RBP1 (Fig. 1, *C* and *D*). Negative controls (co-IP using anti-901 in vector-transfected cells or co-IP using an irrelevant antibody, anti-Lamin A/C) did not pull down RBP1 (Fig. 1, *C* and *D*). Antibody directed against RBP1 co-immunoprecipitated BRMS1 in both breast carcinoma (Fig. 1*E*) and melanoma (Fig. 1*F*) cells.

To begin defining the binding domains of BRMS1 responsible for interactions with RBP1, three C-terminal deletion mutants of 901-tagged BRMS1 were generated by exonuclease III digestion, designated BRMS1( $\Delta$ 204–246), BRMS1( $\Delta$ 164–246), and BRMS1( $\Delta$ 91–246) (Fig. 2*C*). Deletion constructs were transfected into both MDA-MB-231 and C8161.9. The latter expressing clones were experimentally more useful, because expression of all three deletion mutants was approximately equivalent to full-length protein (data not shown, but can be inferred from Fig. 2*B*). In MDA-MB-231, only BRMS1( $\Delta$ 204–246)-expressing clones had protein levels approximating full-length BRMS1 (inferred from Fig. 2*A*). Anti-901 antibody was used to co-immunoprecipitate deletion mutants, and immunoblotting was used to detect RBP1 (Fig. 2, *A* and *B*). Loss of amino acids 204–246 did not decrease binding to RBP1 in either cell line (Fig. 2, *A* and *B*). Loss of amino acids 164–246 diminished binding (by ~90% by densitometry), and loss of amino acids 91–246 abrogated binding (Fig. 2*B*). Absence of binding by BRMS1( $\Delta$ 91–246) was controlled internally for nonspecific binding of RBP1 to the 901 epitope. Interestingly, in both MDA-MB-231 and C8161.9, BRMS1( $\Delta$ 204–246) co-immunoprecipitated RBP1 more effectively (~1.5-fold) than full-length BRMS1 (Fig. 2, *A* and *B*).

**BRMS1 Does Not Appear to Complex with Rb or p107 or to Modulate E2F-dependent Gene Expression**—RBP1 binds Rb family members p105 (RB) and p107 (30–32). Rb proteins, in turn, bind E2F and tether RBP1 to E2F-responsive gene promoters. In this way, RBP1 directly suppresses transcription. We tested the hypothesis that BRMS1 is part of an RBP1-Rb-E2F complex; however, BRMS1 did not co-immunoprecipitate p105 or p107 in MDA-MB-231 (Fig. 2*A*) or C8161.9 cells (data not shown). Likewise, BRMS1 did not affect luciferase expression using an E2F-responsive promoter (data not shown). Taken together, these findings suggest that BRMS1 does not act as part of an RBP1-Rb-E2F complex and that BRMS1 might be part of a previously undescribed RBP1 complex that does not contain Rb.

**BRMS1 Co-immunoprecipitates Several <sup>35</sup>S-labeled Proteins in MDA-MB-231**—Anti-901 was used to co-immunoprecipitate BRMS1 from <sup>35</sup>S-labeled lysates from BRMS1-transfected MDA-MB-231. Vector-transfected cells were used as controls. In addition to BRMS1, several additional bands were evident, including prominent large proteins at  $\geq$ 200 kDa, ~160 kDa, and ~65 kDa as well as less intense bands just below 50 kDa and another at ~30 kDa. (Fig. 3). Parallel experiments were performed using BRMS1-transfected C8161.9 and *Brms1* (murine ortholog; Ref. 7)-transfected 66cl4. Similar <sup>35</sup>S-labeled proteins were co-immunoprecipitated by anti-901 (data not shown). The pattern was reminiscent of previously published

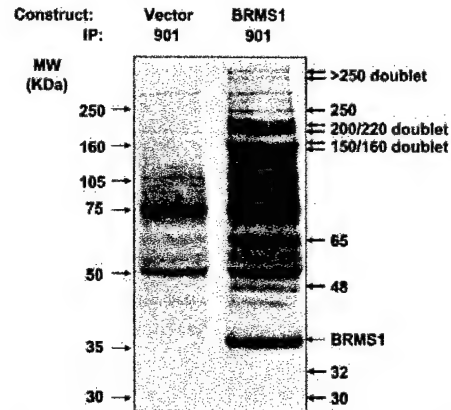




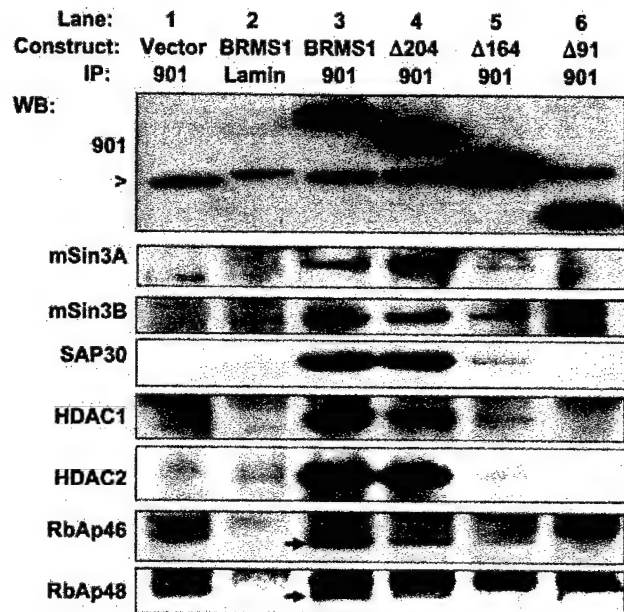
**FIG. 2. Binding to RBP1 is abrogated as C-terminal amino acids are removed from BRMS1, and BRMS1 does not co-immunoprecipitate Rb or p107.** A, whole cell lysates (1 mg) were prepared from MDA-MB-231 cells expressing 901-epitope-tagged BRMS1 or BRMS1(Δ204–246) (see panel C). BRMS1(Δ204–246) co-immunoprecipitated RBP1 (lane 3). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1). To determine relative protein expression, 50 μg of protein lysate from each transfected construct was immunoblotted (lanes 4–6) (the exposure for α-901 shown here was not long enough to show expression in lanes 4 and 5). BRMS1 did not co-immunoprecipitate Rb or p107. IP, immunoprecipitation; WB, Western blot. B, whole cell lysates (1 mg) were prepared from C8161.9 cells expressing BRMS1 and BRMS1 deletion mutants (see panel C) with protein levels comparable with the clone expressing full-length BRMS1. The deletion mutants exhibited varying abilities to co-immunoprecipitate the above-mentioned proteins (lanes 4–6). Anti-901 (lane 1) and an irrelevant antibody (anti-Lamin A/C, lane 2) did not pull down RBP1 in vector-transfected cells. > indicates IgG light chain. C, schematic of BRMS1 deletion mutants. Equal loading of immunoprecipitate is inferred from the data, because equal intensity is observed by probing with anti-901.

results showing that RBP1 interacts with the mSin3-HDAC complex (31, 32). Specifically, HDAC1 and HDAC2 migrate at ~65/60 kDa. mSin3B and mSin3A migrate at ~160/150 kDa. These molecular mass proteins corresponded to the most prominent radiolabeled proteins co-immunoprecipitated with BRMS1 (Fig. 3). Therefore, we hypothesized that BRMS1 is a component of the mSin3-HDAC complex.

**BRMS1 Is a Component of the mSin3-HDAC Complex in C8161.9 and MDA-MB-231—**Immunoprecipitation of epitope-tagged BRMS1 followed by immunoblotting showed that BRMS1 pulled down seven proteins shown previously to be part of mSin3-HDAC complexes, namely mSin3A, mSin3B, HDAC1, HDAC2, SAP30, RbAp46, and RbAp48 (Fig. 4). The same proteins were not precipitated in vector-transfected cells (Fig. 4, lane 1), nor were they pulled down using an antibody to

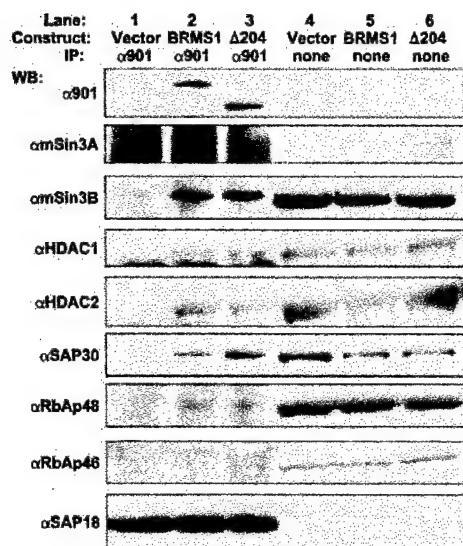


**FIG. 3. BRMS1 co-immunoprecipitated several proteins using <sup>35</sup>S-labeled whole cell lysates.** Using radiolabeled protein lysate from MDA-MB-231 cells, anti-901 was used to immunoprecipitate epitope-tagged BRMS1. Immunoprecipitation (IP) of BRMS1 revealed at least 12 co-immunoprecipitated proteins. Arrows with numbers indicate co-immunoprecipitated proteins and approximate molecular mass (MW) in kDa. > indicates IgG light chain.



**FIG. 4. BRMS1 co-immunoprecipitated at least seven members of the mSin3 HDAC complex in C8161.9 human melanoma cells.** A, BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp46, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected C8161.9 cells (lane 3). Whole cell lysates (1 mg) were also prepared from C8161.9 cells expressing BRMS1 deletion mutants (see Fig. 2C) with protein levels comparable with those of the clone expressing full-length BRMS1. Deletion mutants exhibited varying abilities to co-immunoprecipitate the above-mentioned proteins (lanes 4–6). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1), and anti-Lamin A/C (an irrelevant antibody) did not pull down these proteins in BRMS1-transfected cells (lane 2). IP, immunoprecipitation; WB, Western blot.

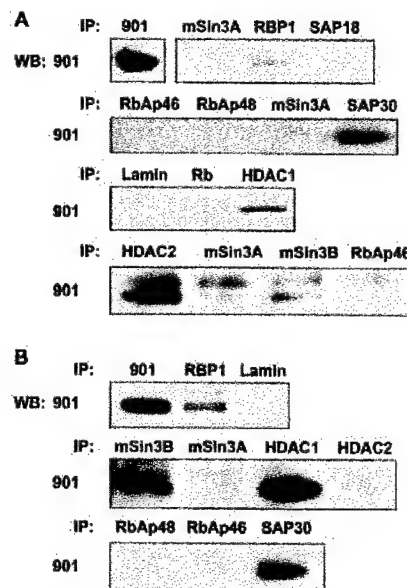
the nuclear protein Lamin A/C (Fig. 4, lane 2). Western blots demonstrated that BRMS1-associated proteins were present at comparable levels in both vector- and BRMS1-transfected cell lysates (data not shown), ruling out the possibility that vector-transfected cells had lower levels of mSin3-HDAC complex components. Interactions between BRMS1 and mSin3-HDAC were relatively strong, because they persisted in 0.5 M NaCl. Antibodies recognizing mSin3B, HDAC1, HDAC2, and SAP30 “reverse” co-immunoprecipitated BRMS1 in C8161.9 cells as well (Fig. 6A).



**FIG. 5. BRMS1 co-immunoprecipitated at least six members of a mSin3 histone deacetylase co-repressor complex in MDA-MB-231 human breast carcinoma cells.** A, BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected MDA-MB-231 cells (lane 2). Whole cell lysates (1 mg) were also prepared from MDA-MB-231 cells expressing BRMS1 deletion mutant ( $\Delta$ 204–246) (see Fig. 2C) with protein levels comparable with the clone expressing full-length BRMS1. BRMS1( $\Delta$ 204–246) also co-immunoprecipitated the above-mentioned proteins (lane 3). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1). To determine relative protein expression, 50  $\mu$ g of protein lysate from each transfected construct was immunoblotted (lanes 4–6) (the exposure for  $\alpha$ -901 was not long enough to show expression in lanes 4 and 5). IP, immunoprecipitation; WB, Western blot.

mSin3-HDAC complex proteins exhibited the same general interaction pattern with BRMS1 deletion mutants as did RBP1, with some exceptions. BRMS1( $\Delta$ 204–246) co-immunoprecipitated mSin3A, mSin3B, SAP30, and HDAC2 at levels comparable with full-length BRMS1 (Fig. 4). However, BRMS1( $\Delta$ 204–246) co-immunoprecipitated HDAC1, RbAp46, and RbAp48 less efficiently than full-length BRMS1 (reduced ~40% by densitometry) (Fig. 4). This discrepancy is evident on co-IP/immunoblots simultaneously probed for HDAC1 and mSin3B, clearly demonstrating differential binding (data not shown). BRMS1( $\Delta$ 164–246) co-immunoprecipitated all mSin3-HDAC complex components significantly less efficiently than full-length BRMS1 (reduced ~90% by densitometry), whereas BRMS1( $\Delta$ 91–246) did not co-immunoprecipitate any complex proteins (Fig. 4).

To determine whether BRMS1 interacted with mSin3-HDAC complex proteins in human breast cancer cells, proteins were co-immunoprecipitated from BRMS1-transfected MDA-MB-231. Six mSin3-HDAC complex proteins, mSin3A, mSin3B, HDAC1, HDAC2, SAP30, and RbAp48 (Fig. 5), were pulled down with BRMS1. Co-IP in vector-transfected cells did not co-immunoprecipitate these proteins (Fig. 5, lane 1) despite the proteins being present in both vector- and BRMS1-transfected lysates (Fig. 5, lanes 4 and 5). As above, interactions persisted in 0.5 M NaCl. RbAp46, a member of the core mSin3-HDAC complex, did not co-immunoprecipitate with BRMS1 in MDA-MB-231 cells (Fig. 5). Antibodies recognizing mSin3B, SAP30, HDAC1, and HDAC2 co-immunoprecipitated BRMS1 in MDA-MB-231 (Fig. 6B). BRMS1( $\Delta$ 204–246) co-immunoprecipitated mSin3-HDAC proteins at levels comparable with full-length BRMS1 (Fig. 5). In both melanoma and breast carcinoma cells, it was not possible to definitively demonstrate that BRMS1 co-immunoprecipitates SAP18, because SAP18 anti-sera also

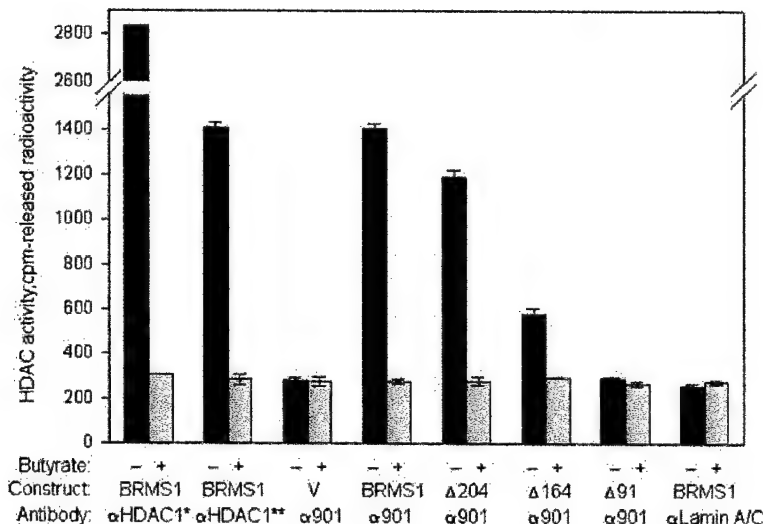


**FIG. 6. HDAC1, HDAC2, SAP30, RBP1, mSin3B, and mSin3A co-immunoprecipitated BRMS1.** A, in BRMS1-transfected C8161.9 cells, antibodies recognizing mSin3A, mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell lysate. Antibodies directed against SAP18, RbAp46, RbAp48, and pRb did not co-immunoprecipitate BRMS1. Presence of the respective antigen recognized for co-immunoprecipitate was confirmed by re-staining the blots with the same antibody used for precipitation. \*, in the bottom panel, increased exposure time was used to reveal co-immunoprecipitated BRMS1, causing a cross-reacting band of slower mobility to become visible. B, in BRMS1-transfected MDA-MB-231 cells, antibodies directed against mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell lysate. Antibodies directed against mSin3A, SAP18, RbAp46, RbAp48, and pRb did not co-immunoprecipitate BRMS1. Anti-901 was used as a positive control. Presence of the precipitated antigen was verified by re-staining the blots with the antisera used for co-IP. IP, immunoprecipitation; WB, Western blot.

recognized a band at ~18 kDa in vector- and BRMS1-transfected cells (Fig. 5).

**BRMS1 Interacts with a Subset of mSin3-HDAC Complexes**—Many proteins that bind HDAC complexes are responsible for recruiting complexes to specific promoters. However, BRMS1 does not have a predicted DNA-binding motif, suggesting that it might serve a different role as a member of subsets of mSin3-HDAC complexes.

As a first step to evaluate those potential roles, the ability of BRMS1 to co-immunoprecipitate selected HDAC complex components was tested. Mad and Max were the first proteins shown to recruit the mSin3-HDAC to a specific promoter (33–35), but BRMS1 did not co-immunoprecipitate Mad1 or Max (data not shown). The unliganded nuclear hormone co-receptors SMRT and NCoR have also been reported to recruit the mSin3 (36–39), but there are contradictory data (40). In our system, BRMS1 did not co-immunoprecipitate SMRT or NCoR (data not shown). mSin3-HDAC interaction with MeCP2, a methyl CpG-binding protein, has also suggested that repression associated with DNA methylation may be mediated, in part, by deacetylation (41). Yet, BRMS1 did not co-immunoprecipitate MeCP2 (data not shown). Because the core HDAC subunit (HDAC1, HDAC2, RbAp46, and RbAp48) is also present in the NuRD HDAC complex (16), we asked whether BRMS1 complexed with NuRD. BRMS1 did not co-immunoprecipitate Mi-2 or MTA1, two members of the NuRD complex (data not shown). HDAC3, which is related to HDAC1 and HDAC2 and can complex with RBP1 (32), did not co-immunoprecipitate with BRMS1 (data not shown).



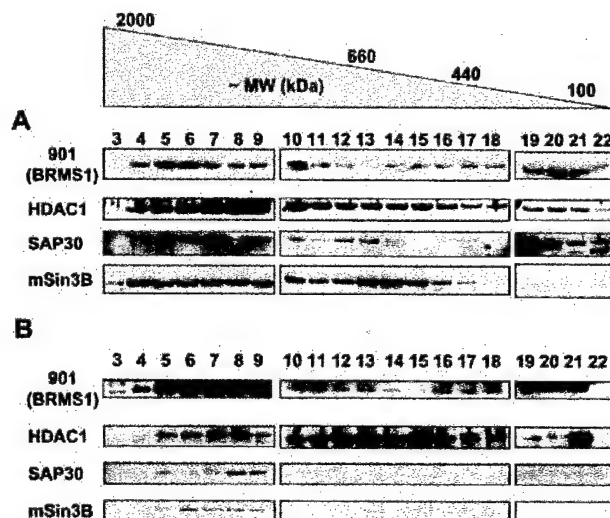
**FIG. 7. BRMS1 pulls down HDAC activity.** Whole cell lysate (6 mg of total protein) was prepared from BRMS1-transfected C8161.9 cells as well as from C8161.9 cells expressing BRMS1 deletion mutants ( $\Delta 204$ ,  $\Delta 164$ , and  $\Delta 91$ ) and vector-transfected (V) cells. Anti-901 was used to immunoprecipitate BRMS1 and BRMS1 deletion mutants from this lysate, and co-immunoprecipitated HDAC activity was measured. The HDAC inhibitor sodium butyrate (250 mM) was used to show that the release of  $^3\text{H}$ -acetyl groups was due specifically to HDAC activity. Anti-HDAC1 was used as a positive control (\*, 10  $\mu\text{g}$  of anti-HDAC1 was used; \*\*, 5  $\mu\text{g}$  of anti-HDAC1 was used). Anti-Lamin A/C was used as a negative control. Bars with error bars represent mean  $\pm$  S.E. for two independent experiments. See Fig. 2C for a schematic of the BRMS1 deletion mutants.

Taken together, these data suggest that BRMS1 exists in a specialized subset of mSin3-HDAC complexes rather than existing as an integral component of the complex. In other words, BRMS1 is not a ubiquitous member of mSin3-HDAC complexes.

**BRMS1 Exists in Large (1.4 and 1.9 MDa) mSin3-HDAC Complexes as Well as Smaller Complexes Containing HDAC1**—To determine the size of BRMS1-mSin3-HDAC complex(es) and the distribution of these molecules in complexes of various sizes, whole cell protein lysates from C8161.9 were subjected to Superose 6 size exclusion chromatography. Fractions were separated by PAGE, transferred to polyvinylidene difluoride, and immunoblotted for 901-BRMS1, HDAC1, SAP30, and mSin3B. These four proteins were chosen because they are core members of the complex. BRMS1 eluted in multiple peaks from the column with complex sizes ranging from  $\sim 100$  to 2,000 kDa. BRMS1 elution was most prominent in peaks 5 and 6 ( $\sim 1.7$  MDa). HDAC1 also eluted in multiple peaks (fractions 4–22) with the majority present in fractions 8 and 9 ( $\sim 1.4$  MDa, Fig. 8A). SAP30 was detected in two peaks, one from fractions 4 through 14 and another from fractions 19 to 24, suggesting the existence of at least two complexes, the first  $>1$  MDa and the second  $<200$  kDa (Fig. 8A). mSin3B is detected uniformly in fractions 3–17, indicating involvement in complexes ranging from  $\sim 2$  MDa to hundreds of kDa (Fig. 8A).

BRMS1 was immunoprecipitated from 420  $\mu\text{l}$  of each fraction followed by PAGE and immunoblot. The vast majority ( $>90\%$ ) of BRMS1 was present in complexes ranging in size between 1.4 and 1.9 MDa (fractions 5–9 shown in lanes 5–9, Fig. 8B). BRMS1 also precipitated in fractions 10–23. HDAC1, SAP30, and mSin3B co-immunoprecipitated with BRMS1 in fractions 5–9, although SAP30 is most abundant in fractions 8 and 9 (lanes 8 and 9, Fig. 8B). HDAC1, however, also co-immunoprecipitated with BRMS1 in fractions 10–21, suggesting that BRMS1 can be involved in smaller complexes with HDAC1 (lanes 10–21, Fig. 8B).

**BRMS1 Co-immunoprecipitates HDAC Activity**—To determine whether BRMS1-associated HDAC1 and HDAC2 were enzymatically active, complexes were assessed for deacetylase activity in C8161.9. Full-length BRMS1 co-immunoprecipitated HDAC activity; BRMS1( $\Delta 204$ –246) pulled down less



**FIG. 8. BRMS1 co-immunoprecipitates a large ( $\sim 1.6$  MDa) complex containing HDAC1, SAP30, and mSin3B as well as smaller complexes containing HDAC1.** A, elution profile of BRMS1, HDAC1, SAP30, and mSin3B in BRMS1-transfected C8161.9 cells. Whole cell lysate (3 mg total protein) was prepared and applied to a Superose 6 size exclusion column. Fractions (500  $\mu\text{l}$ ) were collected, and 20  $\mu\text{l}$  of each fraction were subjected to SDS-PAGE and immunoblotting. MW, molecular mass. B, immunoprecipitation of BRMS1 within eluted fractions. Whole cell lysate (3 mg of total protein) was prepared from BRMS1-transfected C8161.9 cells and applied to a Superose 6 size exclusion column. Fractions (500  $\mu\text{l}$ ) were collected, and anti-901 was used to immunoprecipitate BRMS1 from 420  $\mu\text{l}$  of each fraction. Immunoprecipitated complexes were subjected to PAGE and immunoblotting.

HDAC activity. BRMS1( $\Delta 164$ –246) co-immunoprecipitates still less HDAC activity, whereas BRMS1( $\Delta 91$ –246) pulled down only background activity (Fig. 7). This pattern is reminiscent of the pattern of interaction with HDAC1 seen by immunoblot (Fig. 4). As a positive control, anti-HDAC1 antibodies were able to pull down HDAC activity (Fig. 7) proportionate to the amount of antibody used (*i.e.* when  $2\times$  anti-HDAC1 was used, double the HDAC activity was precipitated). These results show that only a small portion of the HDAC1

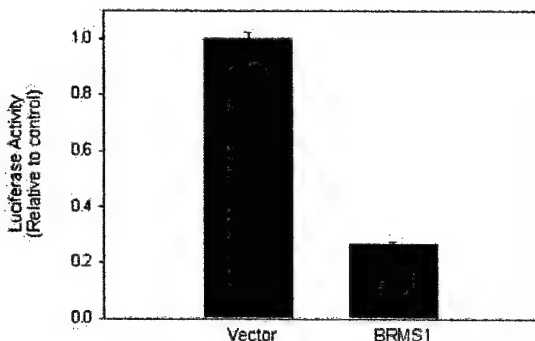


FIG. 9. **BRMS1 represses transcriptional activity *in vivo*.** Using a luciferase reporter assay containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, BRMS1 strongly repressed (~80%) basal transcription compared with the pBIND vector alone.

activity present in the protein lysate is being measured. Vector-transfected cells and co-IP with anti-Lamin A/C served as negative controls (Fig. 7).

**BRMS1 Represses Transcription in Luciferase Reporter Assays**—On the basis of its physical interactions with mSin3 and HDAC1, it was predicted that BRMS1 would repress transcription. To investigate this prediction, we measured the effect of BRMS1 on transcription using a luciferase reporter containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter. BRMS1 strongly repressed (~80%) basal transcription compared with the pBIND vector alone (Fig. 9).

#### DISCUSSION

Epigenetic regulation of the metastatic phenotype was proposed in 1889 when Sir Stephen Paget recognized that tumor cells colonize certain organs preferentially based, in part, upon how they respond to signals from the microenvironment (42). Trainer and co-workers later showed that treatment of murine melanoma cells with the DNA de-methylating agent 5-azacytidine resulted in reversible reduction of metastatic lung colonization (43). Recent studies have shown that treatment of cells with 5-azacytidine can induce expression of the metastasis suppressor genes *Nm23* (44) and *KAI1* (45). Links between metastasis and HDAC activity first became apparent when the breast cancer metastasis promoting gene, *MTA1*, was identified as a component of the NuRD-HDAC complex (46, 47). *MTA1* has subsequently been shown to repress estrogen receptor-dependent transcription in an HDAC-dependent manner (48). Likewise, loss of expression of heterochromatin protein 1 (HP1) has been associated with acquisition of metastatic potential in human breast cancer (49). Together, these findings support the hypothesis that regulation of the transcriptome by a variety of mechanisms is a critical determinant of cancer spread. The findings reported here represent the first direct evidence that a metastasis suppressor gene is a component of an HDAC complex. It is possible that specialized HDAC complexes may promote (as implied by *MTA1*) or inhibit (as implied by BRMS1) cancer metastasis. The data compel the hypothesis that metastasis is regulated, at least in part, by histone deacetylase activity, chromatin remodeling, and/or transcriptional repression.

Connections between HDAC activity and cancer have emerged in recent years, stemming from observations that HDAC inhibitors, such as trichostatin A and suberoylanilide hydroxamic acid (SAHA), can induce growth arrest, differentiation, and/or apoptosis in transformed cultured cells (50). In pre-clinical animal models, HDAC inhibitors have demonstrated impressive anti-tumor activity which, in turn, led to several ongoing HDAC inhibitor clinical trials (50–53). The

data presented here, along with data regarding MTA1 and HP1 cited above, are consistent with the hypothesis that HDAC inhibitors may influence not only primary tumors but also distant metastases.

Interestingly, BRMS1 appears to be part of a protein family in which all of the characterized members are components of the mSin3-HDAC complex. During the original yeast two-hybrid screen, two cDNA clones identified as FLJ00052 were identified in the prostate library. As studies were underway to follow-up RBP1, mSin3, and HDAC findings, FLJ00052 was re-designated by GenBank<sup>TM</sup> as mSds3, the mammalian ortholog of *Saccharomyces cerevisiae* Sds3. Sds3 has been implicated in gene silencing through a Sin3-Rpd3 pathway (Rpd3 in a yeast HDAC1 ortholog) and is an integral component of the yeast Sin3-Rpd3 complex that is required for histone deacetylase activity (17, 54). BRMS1 shares 18% identity and 49% similarity with a large region of yeast Sds3 and 23% identity and 49% similarity with mSds3. mSds3, analogous to its yeast ortholog, is a component of the mSin3-HDAC complex, stabilizes HDAC1 within the complex, and augments HDAC activity (17). Another predicted mammalian protein of unknown function (designated MGC11296) is homologous to both Sds3 and BRMS1. Homology to BRMS1 is particularly strong (58% identity; 79% similarity for the C-terminal 196 amino acids of BRMS1 and the N-terminal 196 amino acids of MGC11296). The high level of sequence similarity between these molecules, combined with their associations with mSin3-HDAC complexes, suggests the existence of a BRMS1 family of proteins that may play a crucial role in altering metastasis by regulating the so-called histone code (29, 49).

Although specific role(s) for BRMS1 within mSin3-HDAC complexes remain to be elucidated, the following lines of evidence suggest that the metastasis suppressor may be involved in recruiting and stabilizing HDAC1 and/or modulating HDAC activity. 1) BRMS1 forms small complexes (~100 kDa and greater) with HDAC1 but forms only large complexes (~1.4 to 1.9 MDa) with Sin3B and SAP30 (Fig. 8B). 2) BRMS1 has distinct binding site(s) for the HDAC1-RbAp46/48 core subunit as compared with the rest of the complex (mSin3A, mSin3B, SAP30, HDAC2, and RBP1) as demonstrated by BRMS1-( $\Delta$ 204–246) binding less effectively to HDAC1-RpAp46/48 than does full-length BRMS1; in contrast, BRMS1( $\Delta$ 204–246) binds the remaining complex components as effectively (Fig. 4). 3) The C-terminal 42 amino acids of BRMS1 appear to stabilize HDAC1-RbAp46/48 within the complex, as deletion of these residues specifically compromises binding to these three components (Fig. 4). 4) Both characterized BRMS1 family members (Sds3 and mSds3) are required for optimal HDAC activity, and mSds3 specifically stabilizes HDAC1 within the mSin3 complex.

Although remarkably similar in breast carcinoma and melanoma cell lines, BRMS1-mSin3-HDAC complexes were distinct. RbAp46 complexes with BRMS1 were not detected in MDA-MB-231 (Fig. 5), and the interaction with RbAp48 appeared less robust than in C8161.9 (compare Figs. 4 and 5). Differential binding of BRMS1( $\Delta$ 204–246) to the HDAC1-RbAp46/48 subunit in C8161.9 was not observed in MDA-MB-231 (compare Figs. 4 to 5). At this juncture, it is not possible to distinguish whether the differences are due to cell origin or presence of mutations that abrogate interactions of RbAp46 with BRMS1-mSin3a. BRMS1-transfected MDA-MB-231 cells are suppressed for metastasis less than C8161.9 (40–90 versus 90–100%). It is tempting to speculate that differences in metastasis suppression may be related to a differential interaction between BRMS1 and the HDAC1-RbAp46/48 subunit.

Preliminary data obtained with the BRMS1 deletion mu-



tants reported here are consistent with a correlation between complexes involving BRMS1, mSin3, and HDAC and metastasis suppression. C8161.9.BRMS1( $\Delta$ 164–246) and ( $\Delta$ 91–246) clones (mSin3 interactions severely impaired or lost; Fig. 4) fail to suppress metastasis (data not shown). However, more refined BRMS1 mutants will be required to determine whether binding to the mSin3-HDAC complex is necessary for metastasis suppression. Systematic site-directed mutagenesis of BRMS1 coupled with metastasis assays are underway.

In summary, the metastasis suppressor BRMS1 is shown here to interact with enzymatically active mSin3-HDAC complexes. BRMS1 is also shown to form smaller complexes with HDAC1 and to repress transcription when recruited to a promoter region. Besides defining a milieu in which BRMS1 works within cells, the data presented here imply that specific downstream mediators, regulated in part by HDAC activity, are critical to controlling metastatic behavior. Indeed, preliminary cDNA microarray and proteomic studies have identified a limited number of BRMS1-regulated genes.<sup>2</sup> Understanding the role(s) of BRMS1-mSin3-HDAC complexes in the regulation of gene expression promises to provide insights into metastasis suppression, HDAC-mediated chromatin regulation, and BRMS1 physiology in noncancerous cells.

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## **Identification of Protein Partners of BRMS1**

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**Running title:** Interactors of BRMS1.

Abbreviations: *BRMS1*, Breast cancer metastasis suppressor gene 1; CMF-DPBS, calcium- and magnesium-free Dulbecco's phosphate buffered saline; DME-F12 , mixture (1:1) Dulbecco's -modified minimum essential medium and Ham's F-12 medium; HBSS, Hank's balanced salt solution; SDS sodium dodecyl sulfate; PAGE, poly acrylamide gel electrophoresis; TTBS, Tris-buffered saline with 0.05% Tween 20;

**Key words:** Metastasis suppressor gene, BRMS1, protein-protein interaction, chromatin modulation, Yeast two hybrid.

**Abstract:**

Breast Cancer Metastasis Suppressor 1, (BRMS1), suppresses metastasis of breast cancer and melanoma in nude mouse model studies. It is a nuclear protein with putative protein-protein interaction domains. We report the discovery of proteins identified to be interacting with BRMS1 and their implication in mechanism of action of BRMS1

**1. Introduction:**

Metastasis suppressors is a distinct class of genes that suppress the spread (or growth at secondary site) of cancer cells at a discontinuous site, without affecting the growth of the primary tumor [1]. Breast cancer metastasis suppressor 1 (BRMS1) has been demonstrated to suppress metastasis of breast cancer and melanoma [2,3,4]. It has also been shown to be negatively correlate to balder cancer [5] and hepatocellular carcinoma [6] progression. BRMS1 is highly conserved gene across species [<http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=25855>] and its murine ortholog brms1 has also been shown to suppress murine mammary carcinoma metastasis [7]. Attempts to understand its mechanism of action have revealed that BRMS1 restores gap junctional communications by increasing the connexin 43 and decreasing connexin 32 [8]. BRMS1 protein shows presence of potential protein binding domains such as leucine zippers and coiled coil domains. We have previously confirmed that BRMS1 is

predominantly nuclear [3]. Hence we hypothesized that BRMS1 interacts with other proteins and is a part of a transcription complex. In order to identify protein partners of BRMS1 we used yeast two hybrid screens. In this study we report the identification of interactors of BRMS1, which give clues about possible mechanism of its action through chromatin remodeling complexe(s).

## **2. EXPERIMENTAL PROCEDURES**

### **2.1 *Cell lines, cell culture:***

COS7 is a monkey kidney cell line. All cell lines were cultured in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium (DME-F12), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, Georgia), 1% non-essential amino acids, 1.0 mM sodium pyruvate. Stable transfected cells also received 500 µg/ml G-418 (Life Technologies Inc., Gaithersburg, Maryland). All cells were maintained on 100 mm tissue culture dishes (Corning, NY, USA), at 37°C , with 5% CO<sub>2</sub> in a humidified atmosphere. COS7 cells were passaged at 80-90% confluence using a solution of 2mM EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup> free Dulbecco's phosphate buffered saline (CMF-DPBS).

### **2.2 *Plasmids and Constructs:***

The plasmids containing FLAG-epitope tagged HDAC2 and 3 were a generous gift from the laboratory of S. L. Schrieber [9,10]. The plasmid containing FLAG-epitope tagged



HDAC1,4,5 and 6 were a generous gift from the laboratory of Edward Seto [11].

mSDS3 cDNA was generously gifted by Don Ayer [12], cDNA for BAF57 was gifted by Bernard Weissman [25] and cDNA for Mrj was provided by Ching-Hua Sung [13]. Nmi cDNA from positive clone pACT(breast) library and BRMS1 cDNA was cloned into the constitutive mammalian expression vector pcDNA3 (Invitrogen, San Diego, California) under control of the cytomegalovirus promotor. To detect BRMS1 protein expression, a chimeric molecule was constructed with an N-terminal epitope tag (SV40T epitope 901) [14].

### **2.3 Yeast Two hybrid Screen :**

The yeast two hybrid screen to isolate cDNA encoding BRMS1 interacting proteins was performed essentially as described by [15,16] . We used pDBTrp and cloned entire coding frame of human BRMS1 in frame with GAL4 DNA binding domain to obtain pDB-BRMS1. This construct was used to transform yeast strain AH 109 (*MATa*, *trp-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1<sub>TATA</sub>-HIS3*, *GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2*, *URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>-lacZ*, *MEL1*) (BD Biosciences, Clontech, CA, USA) to obtain a GAL4DB-BRMS1 fusion (bait) expressing strain of AH109. This strain was used to screen mammary gland, prostate and placenta MATCHMAKER™ cDNA libraries in pACT2 (BD Biosciences, Clontech, CA, USA). Transformants were selected by the use of appropriate media (yeast drop out minimal medium lacking histidine, tryptophan and leucine). His<sup>+</sup> colonies were tested for growth

on minimal medium lacking adenine, tryptophan and leucine and  $\beta$ -galactosidase activity as described previously [17]. cDNA plasmids were isolated from each positive yeast clone using Zymopreco™ (Zymo Research, Orange, CA) and sequenced. The interaction was reconfirmed by plasmid loss experiment for the bait as well as prey protein coding vectors. Introduction of the missing partner recovered the growth on minimal medium lacking histidine, tryptophan and leucine. This was also confirmed by growth on minimal medium lacking adenine, tryptophan and leucine and restoration of  $\beta$ -galactosidase activity.

#### ***2.4 Transient Transfection***

Transient transfection studies in COS7 were performed using pcDNA3-901-BRMS1, and the plasmids containing the cDNA of the interactors. The transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA), as per the manufacturers instructions. Briefly, COS7 cells were plated on to 100 mm. tissue culture plates one day before the transfection, to achieve a confluence of 80-90%. The cells were transfected using ~24 ug plasmid DNA/plate under serum free conditions. The serum containing medium was added typically after 4-6 hrs of transfection. The proteins were harvested after 40-48 hr. for co-immuno precipitation studies.

#### ***2.5 Antibodies, Immunoprecipitation and Western blotting***

The following antibodies were used for the work. Anti-human BAF57(Active Motif,

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Carlsbad, CA, USA). Anti-SDS3 (ab364G) (Bethyl Laboratories Inc.(Montgomery, TX, USA). Nmi(N-16) (SC-9482) (Santa Cruz Biotechnology Inc., CA, USA). Anti-Mrj antibody was a obtained from Ching-Hua Sung. Anti-901 antibody was provided by Satvir Tevathia. Anti-FLAG M2 was purchased form Sigma ( ).

For co-immunoprecipitation studies, after transfection with appropriate vectors, cells were washed twice with ice-cold PBS and lysed with 1% Triton X 100 (Sigma) lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2.0 mM EDTA), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 µg/ml aprotinin, 50 mM NaF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 µl/ml of a protease inhibitor cocktail containing 4-(2-aminoethyl)benzensulfonylfluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanido) butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Lysate was passed through a 21 g needle several times, incubated on ice for 2.5 hour, then centrifuged for 10 minutes at 14000 rpm at 4°C to remove insoluble debris. Lysates were then rocked gently in the presence of appropriate antibody (1-2 µl) for 1 hr at 4°C. 20 µl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, CA, USA) were added and lysates were rocked overnight at 4°C. Agarose beads were washed twice with PBS, boined in SDS-sample buffer. The solubilized protein was resolved by SDS-PAGE and transferred to PVDF membrane. Proteins were fixed by air drying for 15 min at room temperature. The membrane was then wetted in methanol, rinsed in distilled water and blocked in a TTBS solution (0.05% Tween-20, 20 mM Tris, 140 mM NaCl, pH 7.6) containing 5% dry non-fat milk

for 1 hr. The 901-tagged BRMS1 was detected using 1:5000 dilution of mouse anti-901 ascites (1:200 for BAF57, mSDS3 and Nmi) for 1 hr at room temperature under constant agitation. Membranes were then washed with TTBS and probed with 1:10,000 dilution of sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech, Buckinghamshire, UK) in a solution of 5% nonfat dry milk/TTBS for 1 hr at room temperature before washing in TTBS. Bound secondary antibodies were detected using ECL™ (Amersham-Pharmacia Biotech) for 30 sec to 10 min.

### **3. Results**

#### **3.1 *BRMS1 binds to multiple proteins***

In order to begin to elucidate the mechanism(s) underlying the ability of BRMS1 to suppress metastasis by identifying proteins that interact with BRMS1, a yeast two-hybrid screen was performed as described in Methods. Full length BRMS1 was used as a bait and human mammary gland (or prostate or placenta) cDNA library as a prey. Eight genetic interactors of BRMS1 were identified. These are Nmi (N-myc interactor) [19], MRJ (Hsj40 related chaperone) [20], CCG1 (TAFII250, a protein essential for progression of G phase) [21], SMTN (Smoothelin- a cytoskeletal protein specific to smooth muscles) [22], mSDS3 (SAP45) (Suppressor of defective silencing or Sin3 associated protein) [12,23], KPNA5 (karyopherin alpha 5) [24], BAF 57 (BRG1 associated factor) [25,26] and RBBP1 (Rb binding protein) [27,28,29] (**Table 1**).

### **3.2 Confirmation of the protein interactions by co-immunoprecipitations.**

In order to confirm the interaction between the various interactors and BRMS1, cDNA of the interactors and BRMS1 were co-transfected in COS7 and the immunoprecipitations were performed by interactor specific antibodies. RBBP1[30], SDS3, BAF 57, Nmi and Mrj were confirmed to interact with BRMS1 (**Figure1**). mSDS3, BAF57 and Nmi interaction was confirmed by reverse co-immunoprecipitation as well (data not shown).

### **3.3 BRMS1 binds to Class I as well as Class II HDACs**

RBBP1 and mSDS3 are members of mSin3-HDAC complex. Our independent studies on analysis of this complex has shown that BRMS1 is a member of mSin3-HDAC complex [30]. mSin3 HDAC complex has HDAC I and HDAC II as core members and these belong to class I HDACs. We wanted to examine the interaction of BRMS1 with other HDACs ie. HDAC3 which also belongs to Class I and representative members of Class II HDACs ie. HDAC 4,5,6. BRMS1 and FLAG epitope tagged HDAC-cDNAs were co-transfected in COS7. The immunoprecipitations by anti-FLAG antibodies (**Figure2a.**) showed BRMS1 co-immunoprecipitation. The reverse co-immunoprecipitation further confirmed the interactions (**Figure2 b**). Although HDAC 4 I.P. signal is less it corresponds to the expression level in the lysate.

#### **4. Discussion**

BRMS1 is a nuclear protein that suppresses metastasis. One of the very well observed phenotype after restoration of BMS1 expression is restoration of gap-junctional communication. BRMS1 protein shows presence of potential protein binding domains. Hence we hypothesized that BRMS1 is interacting with other proteins and is a part of a transcription complex. The Yeast Two hybrid screen showed interesting candidate proteins. RBBP1, mSDS3, BAF57, Nmi are involved in regulation of transcription. CCG1 or TAFII250 is also a transcription factor. This strongly supports our contentions about involvement of BRMS1 in regulation of transcription. (NOTE: We were unable to detect TAFII250 in co-IPs in stable expressors of BRMS1 in metastatic breast cancer cell lines such as MDA-MB-231. Also we could not obtain the cDNA for transient transfection studies.)

RBBP1 is a member of mSin3-HDAC complex. Two independent laboratories have recently demonstrated mSDS3 to be a member of mSin3-HDAC complex. Interestingly mSDS3 is a homolog of BRMS1. Thus BRMS1 seems to belong to a class of molecules that together or independently form multi-molecular complexes to regulate gene expression possibly via mSin3-HDAC complex. More importantly BAF57 is a member of SWI/SNF complex, which is a ATP dependent chromatin remodeling complex.

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Recent work in proteomics has demonstrated a possibility of the HDAC complexes and ATP dependent chromatin dependent remodeling complexes working together. ClassI HDACs (HDAC1,2,3 and 8) are nuclear and ClassII HDACs (HDAC4,5,6,7) are usually cytoplasmic. It is intriguing to see recent reports of ClassI and ClassII HDACs as well as SWI/SNF co- localization in nucleus [31,32,33,34] . Thus it is very tempting to speculate BRMS1 as an adopter involved in bridging multiple regulatory complexes.

#### I am currently working on the other discussion regarding other candidates ie. Nmi, Mrj, Smoothelin etc

**Figure Legends**

**Table1.** Genetic Interactors of BRMS1 in Y2H

**Figure1. Interactors Co-Immunoprecipitate BRMS1mSDS3,**

Nmi, Mrj, BAF57 co-immunoprecipitated BRMS1 from whole cell lysates (1mg) of COS7 after transient co-transfections. Lane 1: beads with no antibody added (Did not pull down BRMS1). Lane 2: Cell lysate (80µg) to check the level of transfected BRMS1. Lane 3: immunoprecipitation using an irrelevant antibody ie.  $\beta$ -actin (Did not pull down BRMS1) . Lane 4: Immunoprecipitation with respective interactor antibody. IP indicates immunoprecipitation WB: Western blot. Western blot was performed using anti 901

epitope (BRMS1) antibody.

**Figure 2.**

**(A) BRMS1 Interacts With Class I and Class II HDACs:**

Class I HDACs, HDAC1 and 2 (represented as H1 , H2) and Class II HDACs HDAC 4,5,6 (represented as H4, H5, H6) were immunoprecipitated by BRMS1 from whole cell lysates (1mg) of COS7 after transient co-transfections. Panel (a) is the cell lysate (50µg) showing levels of various HDACs after transient transfection. Panel (b) BRMS1 immunoprecipitate (IP) detected using anti-FLAG antibody (WB). M indicates lane corresponding to the molecular weight marker

**(B)HDACs Co-immunoprecipitate BRMS1**

BRMS1 was immunoprecipitated by Class I HDACs, HDAC 1, 2 and 3 (represented as H1 , H2, H3) and Class II HDACs HDAC 4,5,6 (represented as H4, H5, H6) from whole cell lysates (1mg) of COS7 after transient co-transfections. The immunoprecipitations (IP) were carried out using anti-FLAG M2 antibody and the BRMS1 was detected using anti-901 (BRMS1) antibody. M indicates lane corresponding to the molecular weight marker.

**Acknowledgment**



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Table1:

	<b>Location</b>	<b>Description</b>
<b>RBBP1</b>	Nuclear	Binds to Rb & mSin3 / HDAC
<b>mSDS3</b> (suppressor of defective silencing )	Nuclear	Member of mSin3 / HDAC
<b>BAF 57</b>	Nuclear	BRG associated, Member of SWI/SNF
<b>CCG1</b> (TAF II 250)	Nuclear	Transcription factor
<b>Nmi</b>	Nuclear/ cytoplasmic	Transcription factor
<b>Mrj</b>	Nuclear	Heat shock protein
<b>SMTN</b>	Cytoplasmic	Actin cytoskeleton, stress fiber
<b>KPNA5</b>	Nuclear/ cytoplasmic	Protein transport



Figure1:

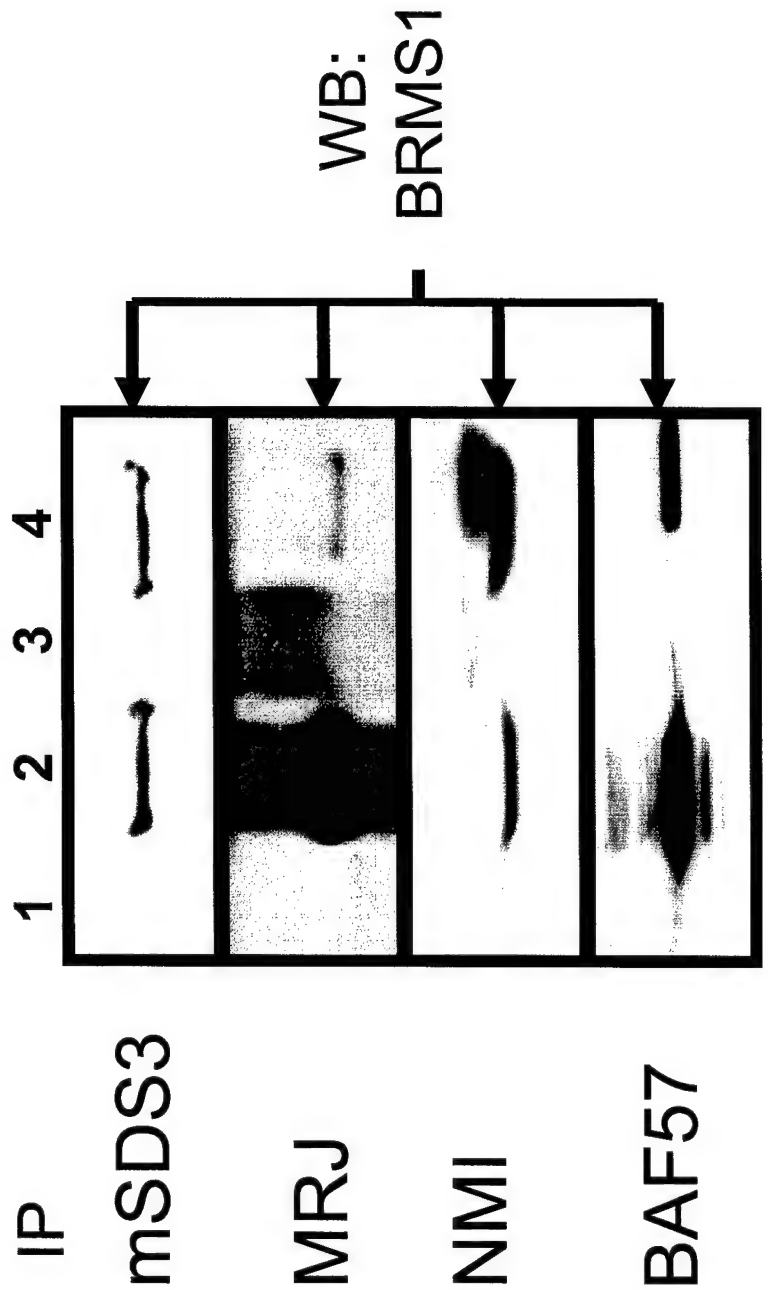
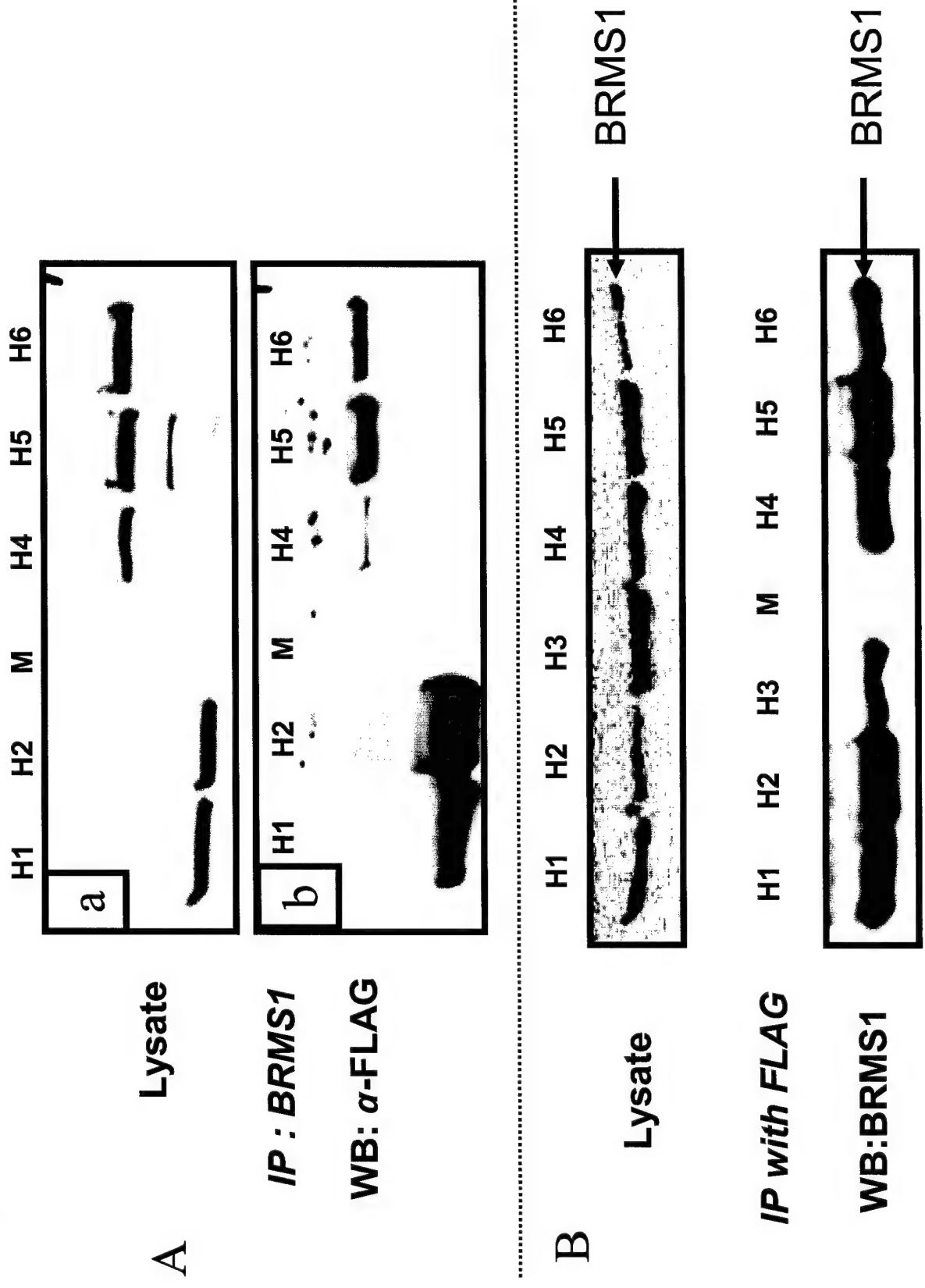


Figure 2:



*Suggested journals:* Cancer Letters,

Mss. No.: \_\_\_\_\_

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**Suppression of murine mammary carcinoma metastasis by murine ortholog of Breast Cancer Metastasis Suppressor 1 (*Brms1*)**

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Running title: Metastasis Suppression by *brms1*.

Abbreviations: *Brms1*, Breast cancer metastasis suppressor gene 1; CMF-DPBS, calcium- and magnesium-free Dulbecco's phosphate buffered saline; DME-F12, mixture (1:1) Dulbecco's - modified minimum essential medium and Ham's F-12 medium; HBSS, Hank's balanced salt solution; SDS sodium dodecyl sulfate; PAGE, poly acrylamide gel electrophoresis, TE, 0.125% trypsin, 2mM EDTA solution in CMF-DPBS; TTBS, Tris-buffered saline with Tween 20; NLS, nuclear localization sequence

Key words: Metastasis suppressor gene, 4T1, breast cancer, murine ortholog

**Abstract:**

**The murine ortholog (Brms1) of human Breast Cancer Metastasis Suppressor 1 shares 95% identity in amino acid structure. We have tested Brms1 for suppression of metastasis of mouse mammary carcinoma cell line 4T1 in syngenic BALB/c mice, using orthotopic (mammary fat pad) implants as well as ectopic (intravenous) injection. As observed for human BRMS1, transfection with *Brms1* did not inhibit 4T1 primary tumor formation, but significantly suppressed its lung colonization. We also show that Brms1 interacts with histone deacetylases, indicating involvement of Brms1 in murine Sin3-HDAC complex.**

**INTRODUCTION**

Metastasis suppressors are relatively newly described group of genes which, by definition, suppress the spread of the primary tumor to a discontinuous site without prohibiting its growth. BRMS1 is located on chromosome 11q13.1-13.2, a region implicated in breast tumor progression to metastasis. Similarly Brms1 is located at a syntenic location and series of genetic screen in mice (Kent Hunter) have implicated this locus as highly linked to metastatic phenotype. Transfection of full-length BRMS1 cDNA into metastatic breast cancer cell lines (MDA-MB-435 and MDA-MB 231) and melanoma (C8161) cell line suppressed metastatic ability without affecting tumorigenicity. Recently we showed that BRMS1 associates with mSin3: Histone deacetylase complex, providing an early clue regarding mechanism of action. The purpose of the present study was to test whether Brms1 exerts a similar metastasis suppression effect and whether comparable protein-protein interactions exist. We show that Brms1 behaves similar to its human counter part,

## MATERIALS AND METHODS

### *Cell lines*

4T1 is derived from a spontaneous carcinoma in BALB/cfC3H mouse [Miller et al., 1980; Aslakson and Miller, 1992; Lelekakis et al., 1999]. Transfectants were derived following transfection of full-length *Brms1* cDNA cloned into a constitutive mammalian expression vector (see below). All the cell lines were cultured in a 1:1 mixture of Dulbecco's-modified minimum essential medium and Ham's F-12 medium (DME-F12), (MediaTech, Cellgro, VA, USA), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA), 1% non-essential amino acids, 1.0 mM sodium pyruvate, but no antibiotics or antimycotics. Transfected cells also received 500 µg/ml Geneticin (G-418) (MediaTech, Cellgro, VA, USA). Cell cultures were maintained on 100-mm Corning tissue culture dishes, at 37°C, with 5% CO<sub>2</sub> in a humidified atmosphere. When cultures reached 80-90% confluence, they were passaged using 2 mM EDTA in Ca<sup>+2</sup>/Mg<sup>+2</sup>-free Dulbecco's phosphate buffer saline (CMF-DPBS). Cells could be routinely split at ratios of 1:10-1:20. *In vitro* doubling times for all cells were between 24-36 hr. Hybrid clones and transfectants were used before passage 14 in order to minimize the impacts of clonal diversification and phenotypic instability [Nicolson, 1987]. For all functional and biological assays, cells between 70-90% confluence were used, with viability > 95%. All the lines were routinely checked and found negative for *Mycoplasma spp.* contamination using GenProbe method (TaKara)

### *Transfection*

Transient transfection studies in 4T1 were performed using pCMV-myc-brms1 the plasmid was

constructed by cloning *Brms1* ORF in frame with the N-terminal myc epitope tag in pCMV-myc (BD-Clontech, CA, USA). The transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA), as per the manufacturer's instructions. Briefly, 4T1 cells were plated onto 100 mm. tissue culture plates one day before the transfection, to achieve a confluence of 80-90%. The cells were transfected using ~24 µg. plasmid DNA/plate. Under serum free conditions. The serum was added typically after 4-6 hrs of transfection. Total protein was harvested after 40-48 hr. for co-immuno precipitation studies.

To generate stable transfectants, *Brms1* was cloned into pcDNA3 (Invitrogen, San Diego, CA, USA) under control of the cytomegalovirus promoter. To detect protein expression, a chimeric molecule was also constructed with an N-terminal epitope tag (SV40T epitope 901) {Fu, Bonneau, et al. 1996 15116 /id} {Kierstead & Tevethia 1993 15117 /id}. Epitope-tagged *Brms1* as well as pcDNA3 vector only were transfected into 4T1 cells by electroporation (BioRad, Hercules, CA; 220V, 960µFd, ∞Ω). Briefly, cells (0.8 ml; 1 x 10<sup>7</sup> cells/ml) from 80% confluent plates were detached using 2 mM EDTA solution. Plasmid DNA (1-20 µg) was added to the cells and the mixture placed onto ice for 5 min before electroporation, followed by 10 min on ice prior to plating onto 100-mm tissue culture dishes. 35 hours later, transfectants were selected by addition of G-418 (500 µg/ml). Single cell clones were isolated by limiting dilution in 96-well plates. Stable transfectants of *Brms1* were assessed for their expression of transcripts by immunoblotting.

### *Immunoblotting*

A monoclonal antibody developed against the 901 epitope of the SV40T antigen amino acids

684-698; was generously provided by Dr. Satvir Tevethia, (Department of Microbiology and Immunology, Penn State University College of Medicine) [Kierstead and Tevethia, 1993; Fu et al., 1996]. Brms1 protein expression was determined by collecting total protein of 70-90% confluent cell cultures. Following aspiration of medium, plates were rinsed 3X with CMF-DPBS before addition of 1 ml lysis buffer (50 mM Tris-HCl, pH 6.8; 2%  $\beta$ -mercaptoethanol, 2% SDS). Lysates were centrifuged at 10,000 x g at 4°C for 15 min to remove insoluble material. Protein concentration was determined using the Bradford method. Protein (20-30  $\mu$ g/lane) was mixed with 5X loading buffer (50% glycerol, 1.5% bromophenol blue) and separated by 12.5% SDS-PAGE. Protein was transferred to Poly Screen® membrane (NEN-Dupont) by semi-dry transfer (5.5 mA/cm<sup>2</sup>, 20V, 30 min). Proteins were fixed by air drying for 15 min at room temperature. The membrane was then wetted in methanol, rinsed in distilled water and blocked in a TTBS solution (0.05% Tween-20, 20 mM Tris, 140 mM NaCl, pH 7.6) containing 5% dry non-fat milk for 1 hr. The 901-tagged BRMS1 was detected using 1:5000 dilution of mouse anti-901 ascites for 1 hr at room temperature under constant agitation. Membranes were then washed with TTBS and probed with 1:10,000 dilution of sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech, Buckinghamshire, UK) in a solution of 5% nonfat dry milk/TTBS for 1 hr at room temperature before washing in TTBS. Bound secondary antibodies were detected using ECL™ (Amersham-Pharmacia Biotech) for 30 sec to 10 min.

#### *Metastasis Assays*

Immediately prior to the injection, cells (7-14 passages following transfection) at 80-90% confluence were detached with 2 mM EDTA solution, washed with CMF-DPBS, counted using a

hemacytometer and resuspended in ice-cold HBSS to a final concentration of  $1 \times 10^6$  cells/ml and 100  $\mu$ l volume was injected in mammary fat pads of female BALB/c mice (5-6 wk age). Tumor size was measured weekly by taking orthogonal measurements and was expressed as mean tumor diameter. Mean tumor diameter was calculated as described by taking the square root of the product of orthogonal measurements [Welch, 1997]. The mice were killed when mean tumor diameter was  $\sim 1.2$  cm.

For the experimental metastasis assay, cells prepared as explained above were injected in to lateral tail vein ( $5 \times 10^5$  cells/0.1 ml), using a 27 gg needle affixed to a 1 cc tuberculin syringe. Mice were killed 19 days post-injection and examined for the presence of metastasis. Lungs were removed, rinsed in water and fixed in diluted Bouin's solution (20% Bouin's fixative in neutral buffered formalin) before quantification of surface metastasis as previously described [Welch, 1997].

Animals were maintained under the guidelines of the National Institute of Health and the Pennsylvania State University College of Medicine. All protocols were approved by Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*.

HDAC activity assay: \*\*\* (Doug /Blake)



**Results***Obtaining Brms1 expressing clones of 4T1:*

We previously found that Brms1 was expressed at practically undetectable levels in 4T1 compared to its non-metastatic counterpart 67NR.(RSS, MTD IJC ) Eepitope tagged murine Brms1 was transfected into 4T1 by electroporation.alongwith corresponding pcDNA3 obtain the vector control. Brms1 expression was tested by western blotting (Figure 1) using anti-901 antibody. Highly expressing clones 6, 10 and 11 were chosen for further studies.

*brms1 suppresses metastasis but not Tumor growth.*

To determine if BRMS1 transfectants alter the rate of primary tumor growth,  $1 \times 10^6$  cells/ml and 100 $\mu$ l volume was injected in mammary fat pads of female BALB/c mice (5-6 wk age). The tumor growth was monitored weekly by orthogonal measurements and was expressed as mean tumor diameter. Mean tumor diameter was calculated as described by taking the square root of the product of orthogonal measurements [Welch, 1997]. The results showed no adverse effect of Brms1 on the tumor growth. (Figure2).

*Brms1 suppresses metastasis of 4T1*

Brms1 transfectant clones of 4T1 were compared with the empty vector as well as parental 4T1 cell line for their ability to metastasize. The metastatic potential was evaluated from inoculation of the cells at the orthotopic (mammary fat pad) site, as well as via lateral tail vein. The Brms1 transfectants showed significantly less metastatic capacity in both the assays (figure 3).

*Brms1 interacts with members of murine mSin3-HDAC complex*

Human BRMS1 was demonstrated as a part of mSin3-HDAC complex. We decided to explore the possibility of Brms1 as a part of a similar murine complex. The co-immunoprecipitation of murine HDAC1, Sin3A, RbAp46 and 48 shows possibility of similar complex operating in mouse. We also confirmed the interaction of Brms1 with the HDACs by the HDAC activity assay. (To be completed)

## DISCUSSION

BRMS1 is a gene widely expressed in both murine and human tissues and is conserved across species. We are interested in analyzing the murine ortholog of BRMS1 (*Brms1*) for the eventual purpose of generating a knockout mouse model for the study of mammary cancer, that will be relevant to breast cancer. The data generated in the series of experiments presented here supports this objective. BRMS1 and *Brms1* are similar in more than one aspects. At the cDNA and protein levels, homology between these two species is significant, 85% and 95%, respectively. The most compelling similarity evident from this study is that *Brms1* and *BRMS1* both suppress metastasis of mammary tumors without affecting tumor growth.

Human BRMS1 is recently shown to be a part of mSin3-HDAC complex. Our studies indicate

that Brms1 is involved in similar murine complex. Since Brms1 also co-immunoprecipitated the HDAC activity, possible involvement of Brms1 in some aspect of histone code based gene regulation in mice is evident. Though at this point the study can't conclude the exact nature of gene regulation that is relevant to metastasis suppression, the knockout mouse for Brms1 will provide a perfect tool to analyze the modification of mSin3-HDAC complex by depleting the Brms1 and will show "real life" phenotype.

#### ACKNOWLEDGMENTS

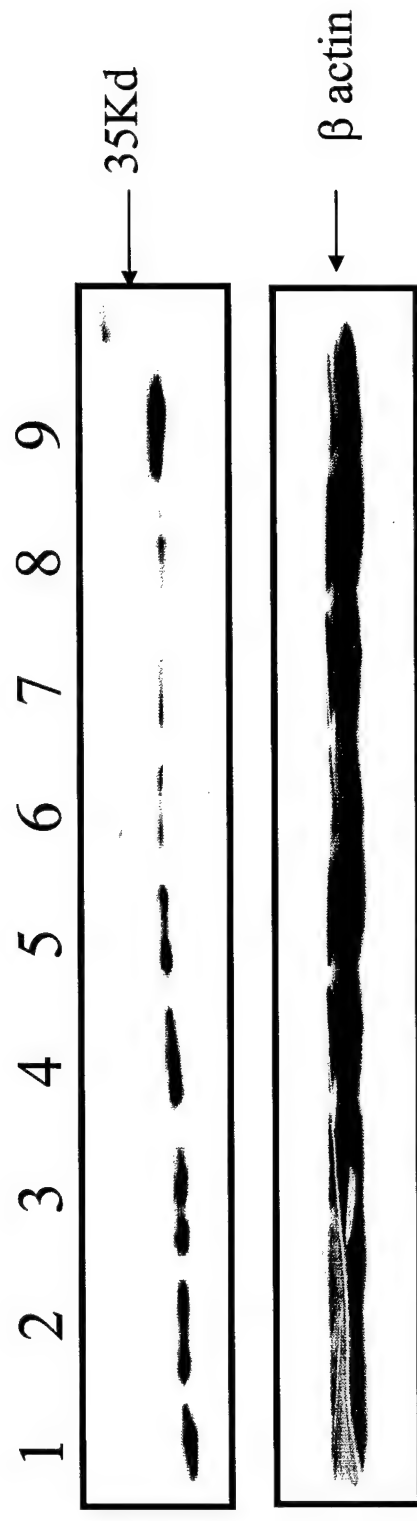
This research was supported by grants to DRW from the U.S. Army Medical Research and Materiel Command DAMD-96-1-6152 and U.S. Public Health Service Grants CA87728 and CA62168. RSS is a recipient of DAMD17-01-1-0362 and UAB Breast SPORE. L.A. Shevde is a recipient of UAB Breast SPORE and IRG 60-001-44 and CA 13148- 31.

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#### REFERENCES

To be added by reference manager.

Figure 1: Confirming Brms1 positive clones



Note: Lane assignment 901 Blot: Lane 1 = cl5, 2 = cl6, etc 9 = + con.

Figure2: Primary tumor growth rate

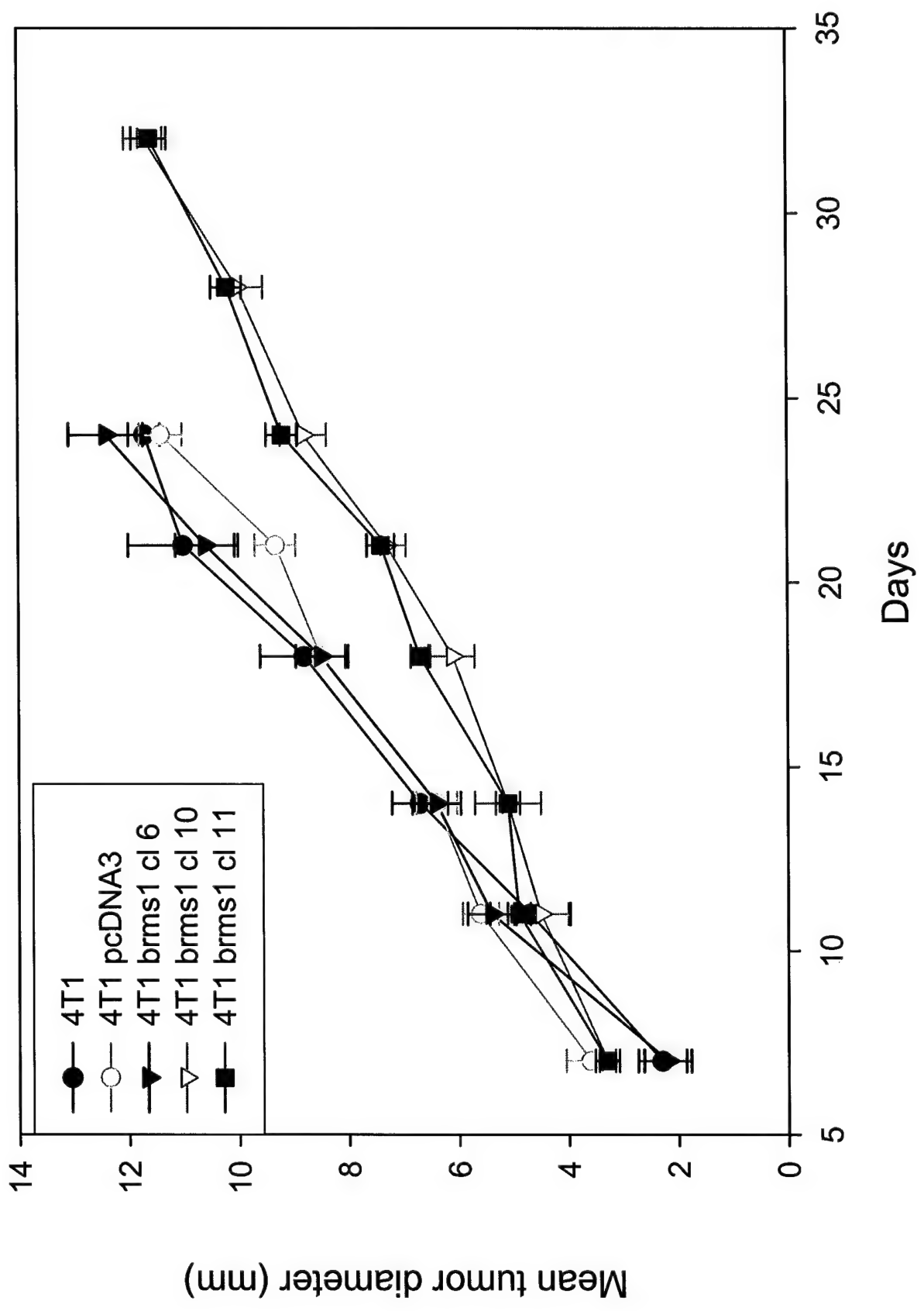


Figure 3 (a): Spontaneous metastasis assay

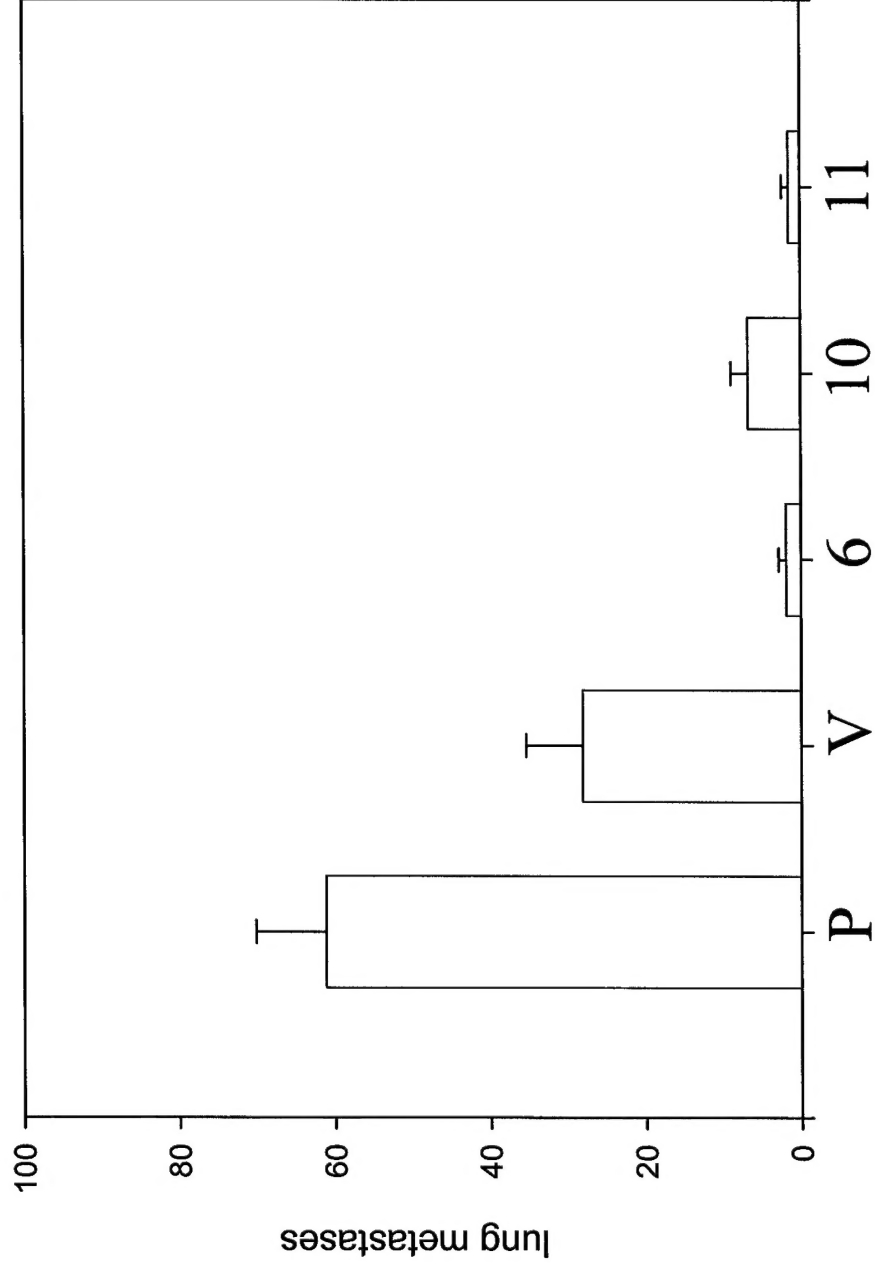


Figure 3 (b) : Experimental metastasis assay

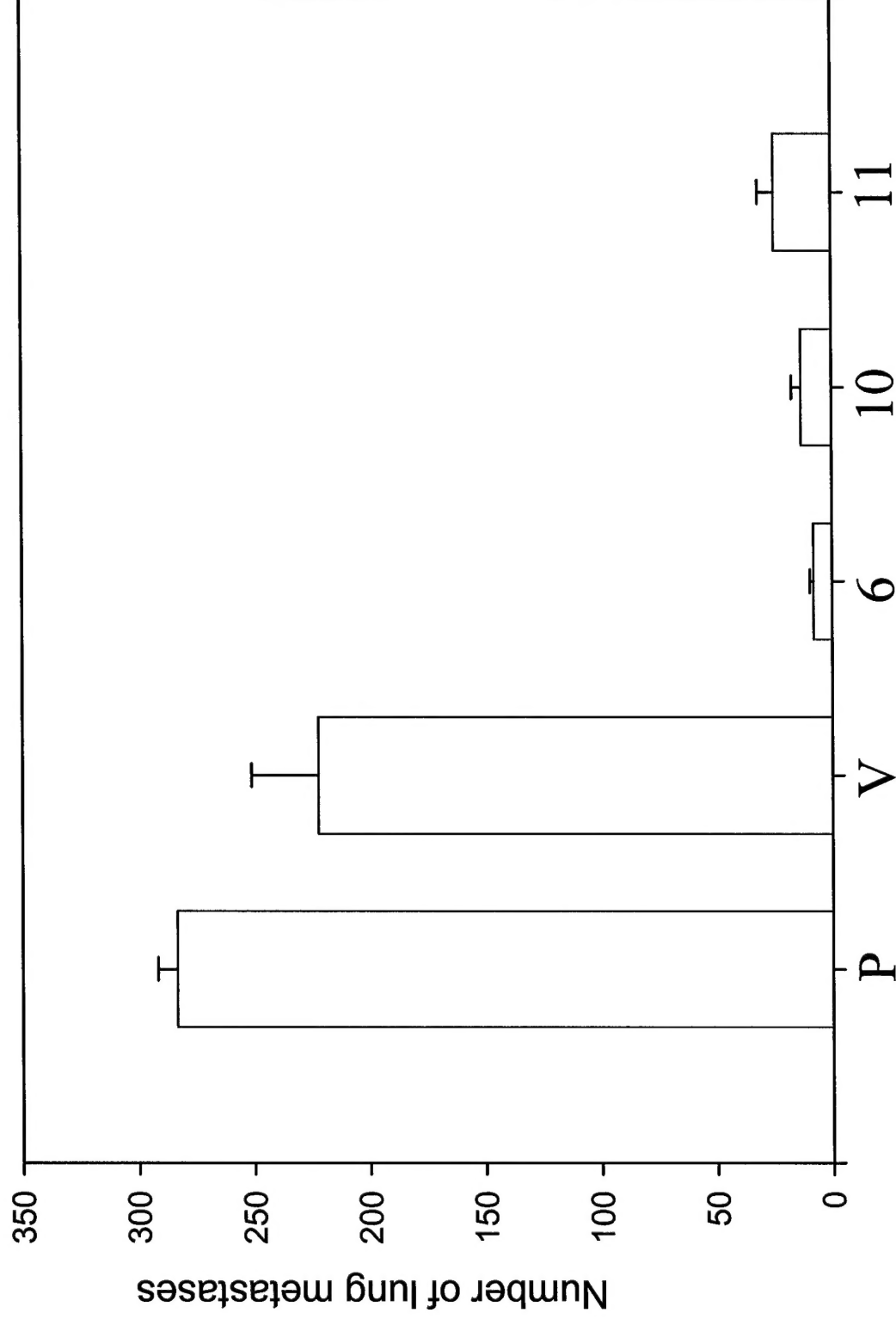
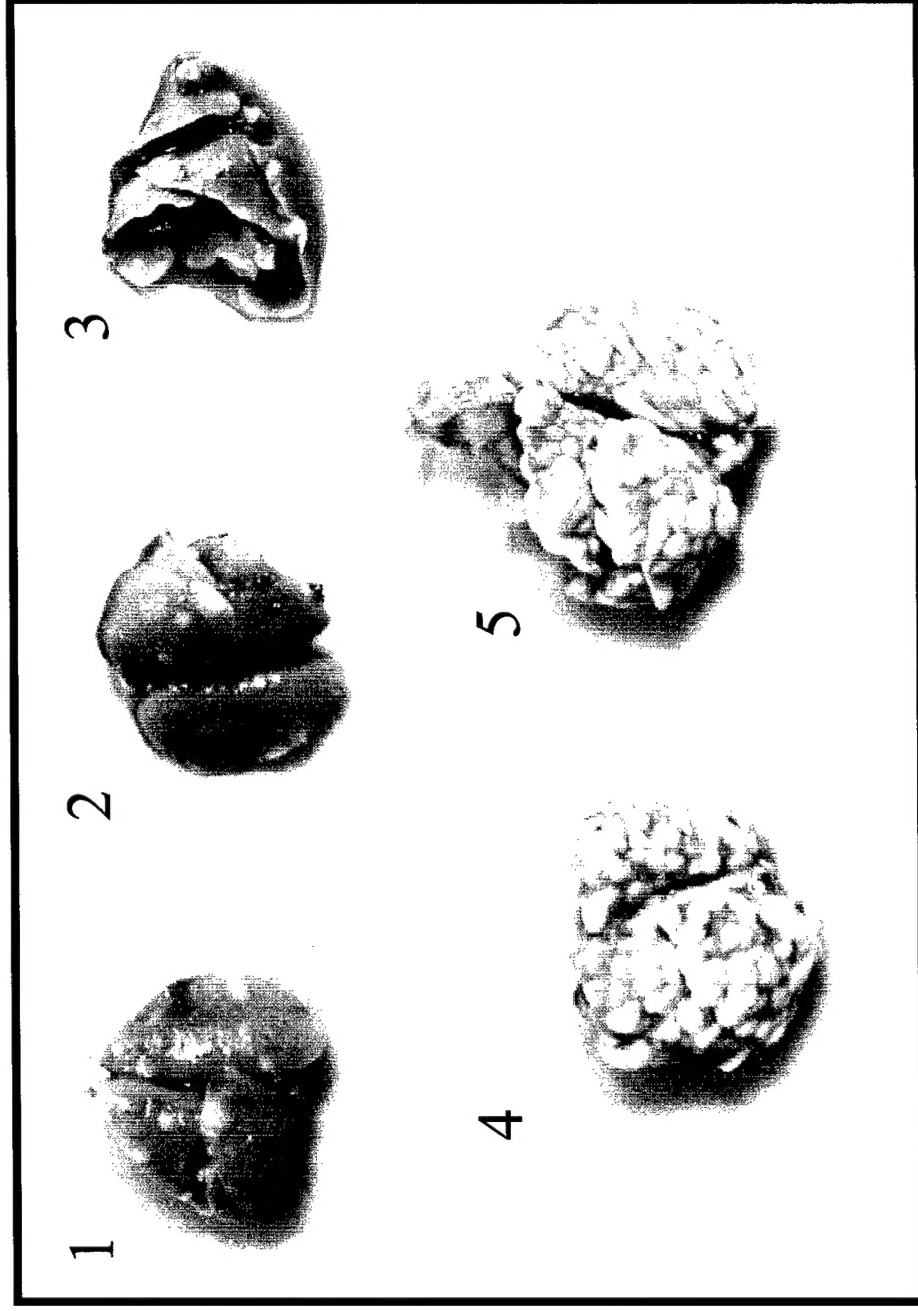


Figure 3 (c) : Photograph of Representative Lungs from 3(b)



- 1 = 4T1 brms1 low  
expressor cl 6
- 2 = 4T1 brms1 high  
expressor cl 10
- 3 = 4T1 brms1 high  
expressor cl 11
- 4 = 4T1 pcDNA3  
vector control
- 5 = 4T1



Table? 4T1 experiments in BALB/C female mice

Cell type	# of mice	# of cells	type of injection	duration (days)
4T1	8	1e5	mfp	32
	9	5e5	ltv	14
4T1 pcDNA3 (pooled)	10	1e5	mfp	32
	11	5e5	ltv	14
4T1 901brms1HA low expressor clone 6	10	1e5	mfp	32
	10	5e5	ltv	14
4T1 901brms1HA high expressor clone 10	9	1e5	mfp	38
	10	5e5	ltv	14
4T1 901brms1HA high expressor clone 11	10	1e5	mfp	38
	10	5e5	ltv	14
	10	2e5	ic	14

mfp= mammary fat pad in 0.2 ml volume  
ltv= lateral tail vein in 0.2 ml volume